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In Vivo Identification of Novel Regulators and Conserved Pathways of Phagocytosis in *A. gambiae*

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Summary

***Anopheles gambiae* uses effective immune responses, including phagocytosis, to fight microbial infection. We have developed a semiquantitative phagocytosis test and used it in conjunction with dsRNA gene silencing to test the in vivo roles of 71 candidate genes in phagocytosis of *Escherichia coli* and *Staphylococcus aureus*. Here, we show that inactivation of 26 genes changes the phagocytic activity by more than 45% and that two pathways similar to those that mediate apoptotic cell removal in *Caenorhabditis elegans* are used in *A. gambiae* for phagocytosis of microorganisms. Simultaneous inactivation of the identified regulators of phagocytosis and conserved components defining each signaling pathway permitted provisional assignment of the novel regulators to one or the other pathway. Pathway inactivation enhances at least three times the ability of *E. coli* and *S. aureus* to proliferate in the mosquito. Interestingly, mosquito survival is not compromised even if both pathways are perturbed simultaneously.**

Introduction

Phagocytosis plays a central role in immune defense and homeostasis. Insects are attractive model organisms for the study of innate immunity, because they lack the complexity of the vertebrate-restricted adaptive immune responses (Hoffmann et al., 1999). Indeed, genetic and molecular studies of humoral defenses in *Drosophila melanogaster* have greatly contributed to our understanding of innate immunity (Hoffmann, 2003). However, cellular immune responses such as phagocytosis (Elrod-Erickson et al., 2000) have been studied almost invariably in vitro in this model organism and remain less well understood. In fact, in the last 4 years, only two new recognition molecules that contribute to microorganism binding and/or engulfment have been characterized by using S2 *Drosophila* cells (Ramet et al., 2001, 2002): scavenger receptor I (dSR-CI), which facilitates binding of gram-negative and gram-positive bacteria to these cells, and PGRP-LC, a peptidoglycan recognition protein that is involved in their phagocytosis of gram-negative, but not gram-positive, bacteria.

The malaria vector *Anopheles gambiae* and species of mosquitoes mount powerful immune responses, including phagocytosis, against a wide range of microorganisms (Hillyer et al., 2004; Levashina et al., 2001). The successful application of double-stranded RNA interference (dsRNA) to silence *A. gambiae* gene expression in vivo (Blandin et al., 2002) and the sequencing of this mosquito's genome (Holt et al., 2002) have opened unprecedented opportunities to dissect immune responses at the molecular level in living mosquitoes. Two recent reports have identified genes in *A. gambiae* that control infections by a *Plasmodium* parasite (Blandin et al., 2004; Osta et al., 2004). Intriguingly, one of these genes, *TEP1*, was shown previously to regulate phagocytosis in mosquito cells in vitro (Levashina et al., 2001).

Here, we report the development of a semiquantitative phagocytosis test inspired by a previous study in *D. melanogaster* (Elrod-Erickson et al., 2000). By using this technological advance in combination with in vivo dsRNA gene silencing and bacterial injections, we have identified unexpected regulators of phagocytosis and have begun to chart the pathways to which they belong.

Results

Development of a Semiquantitative Assay to Monitor Phagocytosis In Vivo

We used in vivo time-lapse microscopy to follow the distribution and fate of fluorescently labeled *E. coli* and *S. aureus* bacteria injected into live adult mosquitoes (Figure 1). Injected bacteria spread almost instantly throughout the mosquito and later form discrete clusters, indicative of association with insect blood cells (hemocytes), which frequently occur in close proximity to the tracheal system (Danielli et al., 2000; Levashina et al., 2001). This association was confirmed by observation of dissected mosquitoes (see the Supplemental Data available with this article online). To quantify and statistically analyze the temporal dynamics of phagocytosis, we designed an integrated image acquisition and processing system, including a dedicated software program (see Experimental Procedures and Supplemental Data). The results allowed us to establish 30 min postinjection as a suitable time point for quantitative analysis of phagocytosis in adult mosquitoes (Figures 2A and 2B).

We next investigated the roles of two genes whose inactivation is routinely performed in our laboratory, *Defensin* (*DEF*) (Blandin et al., 2002) and *TEP1* (Blandin et al., 2004). TEP1 is a thioester-containing hemocyte-

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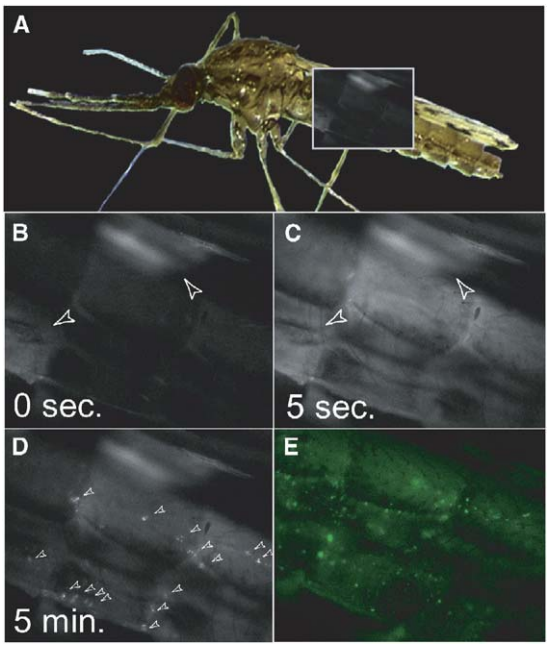

Figure 1. Visualization of *S. aureus* Distribution by Time-Lapse Microscopy

Each mosquito was immobilized in a dish for live microscopy and placed under a 10× objective of an inverted fluorescence microscope. Images were collected from the anterior abdomen as outlined (A). Mosquitoes were injected with fluorescently labeled *S. aureus*, and images were acquired for 5 min and assembled into a video (see Supplemental Data). Representative frames are shown before (B), 5 s (C), and 5 min (D) after bacterial injection. Open arrowheads represent autofluorescence in (B) and (C) and examples of fluorescent clusters in (D). Injection of trypan blue quenches the fluorescence of free and surface-attached bacteria but leaves unchanged the fluorescence of phagocytosed *E. coli* (E) or *S. aureus.*

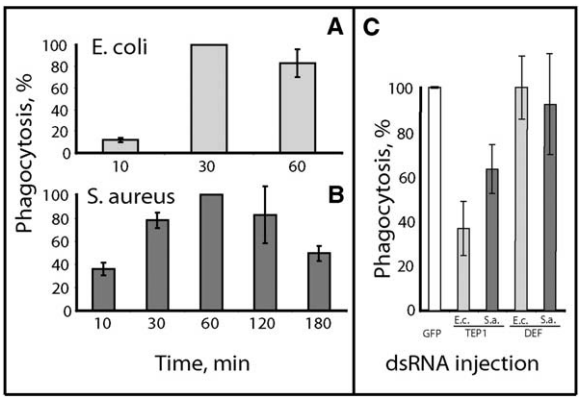

Figure 2. In Vivo Kinetics of *E. coli* and *S. aureus* Phagocytosis

Mosquitoes were injected with either FITC or Alexa488-labeled *E. coli* (A) or FITC-labeled *S. aureus* (B). At the indicated time points, animals were injected with trypan blue and processed for image collection. The highest levels of fluorescence for *E. coli* (30 min) and for *S. aureus* (60 min) were considered as 100% phagocytosis. Error bars represent the SD of the mean from three independent experiments (each experiment is considered the average fluorescence from groups of five to ten mosquitoes). (C) shows the effect of *TEP1* and *DEF* knockdown on *E. coli* and *S. aureus* phagocytosis, and the white bar represents *dsGFP*-injected mosquitoes for both *E. coli* and *S. aureus* phagocytosis.

specific glycoprotein that binds to bacteria in a thioester-dependent manner and promotes phagocytosis of gram-negative, but not gram-positive, bacteria in vitro (Levashina et al., 2001). Conversely, DEF is an antimicrobial peptide that displays bactericidal activity in vitro (Vizioli et al., 2001) and is required for mosquito survival after in vivo infection with gram-positive bacteria (Blandin et al., 2002). We injected 1- to 2-day-old mosquitoes with respective dsRNAs or control green fluorescent protein (GFP) dsRNA and allowed mosquitoes to recover for 4 days. The efficiency of the knockdowns was confirmed in hemolymph samples (Blandin et al., 2002, 2004) as previously reported. We observed no significant change in phagocytic activity in *dsDEF*-treated mosquitoes (Figure 2C). In contrast, *TEP1* was required for optimum phagocytosis in vivo: its knockdown caused ~60% and ~40% decrease in the efficiency of phagocytosis of *E. coli* and *S. aureus*, respectively (Figure 2C). In our previous in vitro studies, the effect on phagocytosis of *S. aureus* was not detected (Levashina et al., 2001), underscoring the importance of the in vivo experiments.

Identification of Novel Regulators of Phagocytosis

We extended this analysis to screen a total of 71 genes. The vast majority of these were included in the screen based on their significant transcriptional regulation after septic injury (Dimopoulos et al., 2002) and are part of an ongoing effort to functionally define the complete repertoire of genes involved in *A. gambiae* innate immune responses. Among the transcriptionally regulated genes, we found the ortholog of a *C. elegans* gene that mediates apoptotic cell removal (*ced-6*). Based on this finding, we designed constructs to target orthologs of other members of the two pathways that are involved in apoptotic body clearance in the worm. A few other genes were tested in light of our previous in vitro work on phagocytosis (Levashina et al., 2001; L.F.M. and F.C.K., unpublished data) to assess their significance in vivo.

4 days postinjection of a standard amount of dsRNAs, levels of phagocytosis were quantified at 30 min after bacterial injection and were compared to the levels observed in mosquitoes injected with control *dsGFP* RNA. In our experience, the efficiency of gene silencing by dsRNA can be estimated reliably only through protein analysis, as the excess dsRNA often interferes with accurate assessment of remaining RNA levels. In our laboratory to date, we have used immunobloting or mass spectrometry assays when possible and thus confirmed specific and highly efficient depletion of silenced gene products in six out of seven cases: *DEF1* and *TEP1* in published studies (Blandin et al., 2002, 2004) and *TEP3*, *TEP4* (S.B. and E.A.L., unpublished data), *LRIM1* (A. Koutsos and F.C.K., unpublished data), and *CTLMA2* in unpublished work. Nothing has been found for *CTL4* (M. Osta and F.C.K., unpublished data). In all six cases, depletion was achieved within 3 days (S.B. and E.A.L., unpublished data). Four of these genes were included in the present study (*TEP1*, *TEP3*, *TEP4*, and *LRIM1*). If the same proportion of successful si-

Table 1. The Inactivation of the Listed 26 Genes Changes Phagocytosis of at Least One Bacterial Species by 45% or More

Category	Gene Name	Ensemble Number	Percentage of Phagocytosis Levels		Score
			E. coli	*S. aureus*	
Chaperones	*HSP70L1*	10404	37.8% ± 5.52%	95.4%	Ec
	HSP20L1	No prediction	37.9% ± 1.63%	151.4% ± 1.3%	Opposite
	HSP20L2	09148	38.1% ± 1.3%	116.9%	Ec
	PPI1	09453	195.6% ± 2.6%	120.5% ± 4.7%	Ec
Enzymes	*SP4A*	16844	33.4% ± 0.4%	137.4%	Ec
	CHI4	02513	41.1% ± 0.99%	119.7%	Ec
	GPIB1	12401	78.1%	147.2% ± 10.9%	Sa
	CPGL	8453	90.7%	163.7% ± 29%	Sa
Protein-protein interactions	*SPEN*	09324	36.3% ± 18%	145.4% ± 32.7%	Opposite
	RANK1	06402	92.8%	50.5% ± 6.4%	Sa
Miscellaneous	*SCLP1*	09597	43.9% ± 17.6%	91.7%	Ec
	FKHL1	19661	46.5% ± 2.8%	108.1%	Ec
	TUSP1	06408	60%	147.1%	Opposite
	IMPB1	08827	75.3%	196.7% ± 1.1%	Sa
	SASB58	No prediction	113%	39.1% ± 12.4%	Sa
	CACT	07525	227.8% ± 23.8%	41.1% ± 14.4%	Opposite
Hemolymph-circulating molecules	*LRIM1*	10552	31.7% ± 8.6%	117.6% ± 3%	Ec
	TEP1	14368	36.9% ± 12%	63.4% ± 11%	Both
	TEP3	13794	52.2% ± 6.6%	104% ± 24%	Ec
	TEP4	18727	36.3% ± 5.7%	44.9% ± 18%	Both
Receptors	*LRP1*	08664	20.5% ± 8%	51.4% ± 8.8%	Both
	BINT2	11759	28.1% ± 10.7%	107.9% ± 8.1%	Ec
	PGRPLC	07834	43.4% ± 2.7%	102.1%	Ec
CED-like	*CED6L*	08628	19.4% ± 15%	49.7% ± 12.4%	Both
	CED2L	08454	45.3% ± 5.7%	71.7% ± 7.3%	Both
	CED5L	02759	45.8% ± 1.9%	44.1% ± 15.5%	Both

The SD is shown for genes with effects greater than 30%. The ensemble number is abbreviated by omitting the prefix ENSANGG000000.

lencing is applicable to the entire set of 71 genes in the present screen, approximately ten of these genes may have been scored falsely as negatives. The use of *dsGFP* RNA as control guarded against false positives.

The effects of gene silencing on phagocytosis of *E. coli* or *S. aureus* were assessed in five to ten individual mosquitoes for each of the 71 genes and for each bacterial species. For 47 genes, we detected a notable change in phagocytosis (30% or more) for one or both bacterial species (see Supplemental Data, Table S1). Additional repeat assays in five to ten mosquitoes were conducted for 26 of these genes (and 38 combinations of gene and bacterial species) where the effects were most pronounced. The combined data confirmed the first-round conclusions and provided reasonable standard deviation (SD) estimates of the experimental variation (Table 1). Interestingly, both enhancement and inhibition of phagocytosis were observed, depending on the gene and the microorganism. For convenience, Table 1 also summarizes our conclusions from the available data, scoring the genes as having effects on phagocytosis of *E. coli* (Ec, 11 genes), of *S. aureus* (Sa, five genes), of both species (both, six genes), or of both species but in opposite directions (opposite, four genes). These 26 genes were ascribed to seven classes according to the nature of the encoded proteins (Table 1). Classes (e)–(g), containing a total of ten genes, are of special interest and are exemplified in Figure 3A.

Genes Encoding Chaperone-like Molecules

Silencing three genes of this class caused a decrease of over 60% in phagocytosis of *E. coli* and, in one case, significantly increased the engulfment of *S. aureus* but otherwise had no effect. Two genes showed homology to the heat shock protein 20 (HSP20) domain, and another was a highly conserved homolog of HSP70 that included the KDEL motif, an endoplasmic reticulum (ER) localization signal. Heat shock proteins are required for efficient protein folding and are often localized in the ER, an organelle that has recently received considerable attention for its role in phagocytosis (Desjardins, 2003). A fourth gene (PPI1) was the ortholog of the *D. melanogaster* gene *ninaA* and encoded a putative peptidylprolyl-*cis-trans* isomerase, mutations of which cause dramatic accumulations of the ER (Colley et al., 1991); this mosquito gene acts as a negative regulator of phagocytosis, especially of *E. coli*.

Enzyme-Encoding Genes

Inactivation of genes encoding a putative serine protease (*SP4A*) and a chitinase (*CHI4*) led to a significant decrease in *E. coli* engulfment. Silencing of two others (*GPIB1*, which has homology to phosphatidylinositol N-acetylglucosaminyltransferases, and *CPGL* with homology to a glutamate carboxypeptidase-like protein) caused a substantial increase in *S. aureus* phagocytosis.

Genes Encoding Protein-Protein Interaction Domains

Silencing *SPEN1*, the ortholog of the *D. melanogaster split ends* gene, caused a strong reduction in phagocytosis of *E. coli*. *Split ends* mutants affect neuronal cell fate and axonal path finding (Kuang et al., 2000); the effects are reminiscent of cell migration defects in mutants of the *C. elegans* genes *ced-2*, *ced-5*, *ced-10*, and *ced-12*, which also affect apoptotic cell engulfment (Su et al., 2002). Another *Anopheles* gene (*RANK1*) encodes ankyrin repeats and an RA domain (IPR000159) often

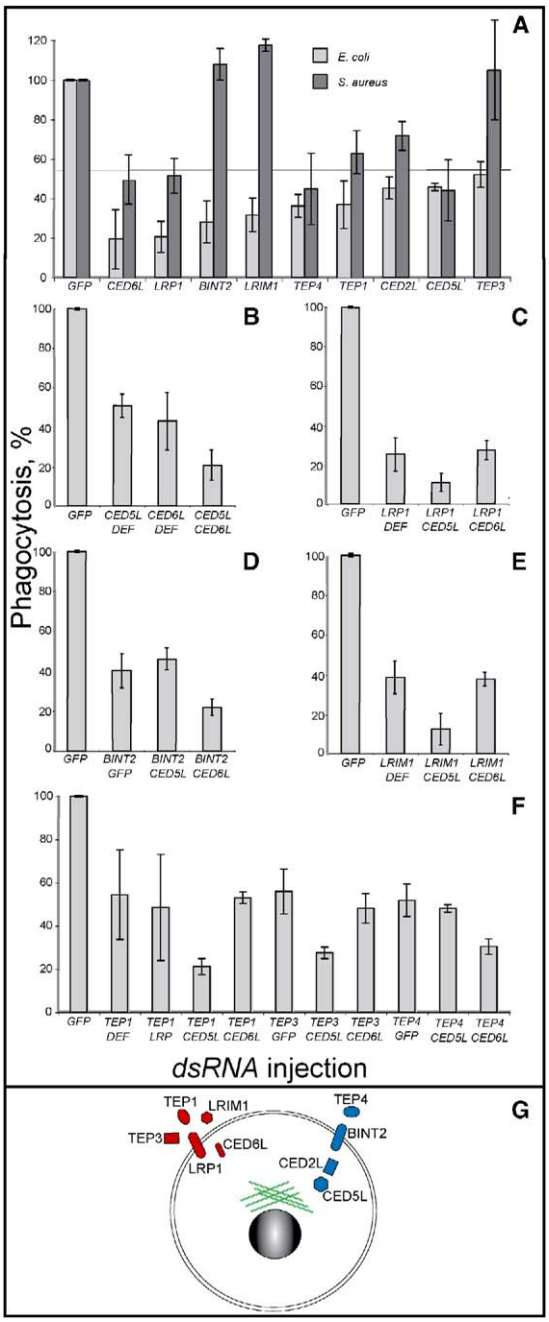

Figure 3. Phagocytosis Measurements in Single Knockdowns of Candidate Genes Showing Significant Effects on Engulfment

Phagocytosis results are presented as a percentage of *dsGFP* controls. Light gray columns represent *E. coli*, dark gray columns represent *S. aureus*, and error bars represent the SD of the mean (each experiment is considered the average fluorescence from groups of five to ten mosquitoes). Results are shown for transmembrane receptors, *C. elegans* CED homologs, and thioester-containing proteins (TEPs) (A). Assignment of genes to different pathways based on phagocytic results in double knockdowns (B–F): double knockdowns of *CED5L* and *CED6L* (B), and placement of *LRP1* (C), *BINT2* (D), *LRIM1* (E), *TEP1*, *TEP3*, and *TEP4* (F) in either the *CED5L* or the *CED6L* pathway. Two major phagocytic pathways regulate phagocytosis of bacteria in *Anopheles gambiae* (G).

associated with RasGTP effectors; its silencing specifically decreases *S. aureus* phagocytosis.

Miscellaneous Genes

Silencing of a novel mosquito gene (*SASB58* or *S. aureus specific clone B58*) specifically decreased *S. aureus* phagocytosis, whereas silencing *FKHL1* (a Forkhead transcription factor homolog) decreased *E. coli* phagocytosis. Silencing *TUSP1* (a Tubby transcription factor homolog) decreased *E. coli* but increased *S. aureus* engulfment. Knockdown of *CACT*, the putative ortholog of *Drosophila* Cactus, an NF-kB transcription factor inhibitor, showed effects opposite to those of *TUSP1.* Finally, silencing *SCLP1*, a homolog of the *Death-associated small cytoplasmic leucine-rich protein* gene of *M. sexta,* decreased engulfment of *E. coli*; in contrast, silencing a homolog of a human importin-β type gene, *IMPBI*, greatly increased phagocytosis of *S. aureus*.

Genes Encoding Hemolymph-Circulating Molecules

The mosquito genome encodes 15 thioester-containing proteins (TEPs), 11 of which were found after the availability of the complete *A. gambiae* genome sequence (Holt et al., 2002). We focused on the functional analysis of our initial subset of four TEP genes, for which we had available transcriptional information and antibodies that permitted confirmation of efficient silencing. The *TEP1* gene encodes a mosquito opsonin that is involved in killing *Plasmodium berghei* ookinetes (Blandin et al., 2004), silencing it significantly reduced phagocytosis of both bacterial species, especially of *E. coli* (Figure 2C). Three additional genes of the *TEP* family were studied: *TEP2*, *TEP3*, and *TEP4*. Silencing *TEP4* significantly affected phagocytosis of both *E. coli* and *S. aureus* by more that 50% in each case, whereas *TEP3* depletion inhibited phagocytosis of *E. coli*, but not *S. aureus* (Figure 3A). In contrast, inactivation of *TEP2* showed no effect on phagocytosis (Table S1).

The *LRIM1* gene (*Leucine-rich Repeat Immune Protein 1*) encodes a homonymous (LRR) domain that is found in adhesive proteins, including Toll receptors. LRIM1 has an extensive array of leucine-rich repeats but is not a member of the Toll family. Like *TEP1*, *LRIM1* is a potent antagonist of *P. berghei*, killing a substantial proportion of midgut-invading ookinetes (Osta et al., 2004). It also displays transcriptional induction after a septic, but not a sterile, injury (Dimopoulos et al., 2002). *LRIM1* knockdown decreased *E. coli* engulfment by almost 70% but did not reduce phagocytosis of *S. aureus*.

Genes Encoding Putative Transmembrane Receptors

LDL receptor-related protein 1 (LRP1) is a large multidomain receptor. The cytoplasmic tail of this protein in vertebrates contains two NPxY motifs that can serve as sorting signals to endosomes and lysosomes as well as docking sites for cytoplasmic adaptor scaffolding proteins involved in signaling events (Herz and Strickland, 2001), for example the phosphotyrosine binding CED-6/GULP protein (Guy et al., 2002; Su et al., 2002). Interestingly, in vertebrates, LRP is the receptor for α_2 macroglobulin (Kristensen et al., 1990), a member of the same family as TEP1 and complement factor C3. The role of human LRP in the engulfment of apoptotic cells has been demonstrated recently (Ogden et al., 2001). The *A. gambiae* LRP has two NPxY motifs in the cytoplasmic domain and is 38% identical and 53% sim-

ilar to mouse LRP. After *LRP1* knockdown, engulfment of *E. coli* decreased approximately by 80% and of *S. aureus* by 50% (Figure 3A). The *BINT2* gene encodes a newly identified member of the β-integrin family that was shown to be important for in vitro phagocytosis of *E. coli* by the hemocyte-like mosquito cell line 5.1* (L.F.M. and F.C.K., unpublished data). In vivo depletion of *BINT2* in mosquitoes caused a reduction of more than 70% in phagocytosis of *E. coli*, but not *S. aureus*. *PGRPLC* belongs to the family of peptidoglycan recognition proteins (PGRPs) characterized by the presence of the PGRP domain (IPR002502), which has central roles in melanization, phagocytosis, and signal transduction leading to the production of effectors against bacteria and fungi. The *A. gambiae* genome contains seven distinct *PGRP* genes (Christophides et al., 2002). Of these, three belong to the short (S) subfamily encoding secreted proteins (*PGRPS1, S2*, and *S3*), and four are part of the long (L) subfamily (*PGRPLA, LB, LC*, and *LD*) and potentially encode transmembrane or intracellular products. As in *Drosophila* where *PGRP-LC* has a role in *E. coli* phagocytosis (Ramet et al., 2002), we observed that silencing of *Anopheles PGRPLC* specifically reduces *E. coli* engulfment by ~60%. We have also tested two additional members of the long *PGRP* subfamily: *PGRPLB and PGGRPLA*. Knockdown of *PGRPLB*, which is transcriptionally regulated in adult mosquitoes by septic injury (Dimopoulos et al., 2002), did not change significantly the phagocytosis of either *E. coli* or *S. aureus*. The knockdown of *PGRPLA* had a similar but less pronounced effect to the knockdown of *PGRPLC* (Table S1), suggesting the possibility that these molecules might cooperate in *E. coli* engulfment.

Homologs of C. elegans Genes Involved in Apoptotic Cell Clearance

In *C. elegans*, apoptotic cells are swiftly removed by neighboring phagocytic cells (Gumienny and Hengartner, 2001). A number of genes have been implicated in this process (Ellis et al., 1991; Hedgecock et al., 1983) and have been grouped genetically into two partially redundant pathways progressively characterized in recent years. Double mutants affecting genes from both pathways cause persistence of much higher numbers of cell corpses than either the strongest single mutants or mutant combinations within the same pathway (Ellis et al., 1991). The first pathway includes *ced-1*, which encodes a scavenger receptor-like transmembrane protein (Zhou et al., 2001), and *ced-7*, which encodes an ABC transporter (Wu and Horvitz, 1998). This pathway also includes ced-6, a protein bearing a phosphotyrosine binding domain (PTB) and a proline/serine-rich segment in the N- and C-terminal regions, respectively (Liu and Hengartner, 1998); the homologous PTB domain-containing human protein GULP binds to LRP and plays a role in the engulfment of apoptotic cells (Ogden et al., 2001). In the second pathway, the *C. elegans* genes *ced-2*, *ced-5*, *ced-10*, and *ced-12*, and their mammalian homologs *CrkII*, *Dock180*, *Rac1*, and *Elmo*, respectively, mediate cytoskeletal rearrangements during phagocytosis of apoptotic cells and cell motility (Gumienny et al., 2001). We first silenced singly the mosquito orthologs of three representative genes. The mosquito *CED6-like* (*CED6L*) ortholog of the *C. elegans* first-pathway gene *ced-6* and of human GULP is strongly upregulated after septic, but not sterile, injury of mosquitoes (Dimopoulos et al., 2002). Its in vivo knockdown resulted in substantially reduced phagocytosis of both *E. coli* and *S. aureus*, much as the *LRP1* knockdown. The knockdowns of the mosquito *CED2L* and *CED5L* genes, corresponding to the second *C. elegans* pathway, also decreased phagocytosis of both *E. coli* and *S. aureus*. However, these effects on *E. coli* engulfment were less prominent than in the case of CED6L.

Provisional Assignment of Genes to the Pathways in which They Operate

In *D. melanogaster*, several genes can be silenced by dsRNA simultaneously without a significant decrease in specificity or efficiency, providing a powerful tool to position genes in alternative pathways (Foley and O'Farrell, 2004; Goto et al., 2003). Therefore, in a new round of experiments, we used epistasis to determine whether multiple mosquito genes could be grouped into potential pathways. In the single knockdowns, we had observed that more genes affected phagocytosis of *E. coli* rather than *S. aureus* and therefore chose to analyze phagocytosis of *E. coli*. We injected each of two dsRNAs at half the amount, so that the double knockdowns would have the same total amount of dsRNA as the previous experiments. Again, levels of observed phagocytosis of *E. coli* were expressed as percentages of the levels in control mosquitoes injected with the corresponding amount of *dsGFP*.

We first confirmed that the half-dose of target dsRNA was also efficient in single knockdowns where the total dsRNA was brought up to the standard level by addition of *DEF* or *GFP* dsRNAs. The results of half-dose and full-dose silencing experiments were comparable, indicating that the half-dose is saturating and is sufficient to interfere with gene function (data not shown). This was confirmed at the protein level (by immunoblot) for TEP1, where a well-characterized antibody is available (S.B. and E.A.L., unpublished data). Therefore, for subsequent experiments, we have assumed that knockdowns are complete, and the phenotypes are not due to two additive hypomorphic phenotypes in the same pathway; we interpreted comparable reduction of phagocytosis in single and double knockdowns as indicating that two tested genes belong to the same pathway and interpreted additive effects as indicating that the genes belong to different pathways.

The inhibitory effects of *CED6L* and *CED5L* dsRNA on phagocytosis were additive: phagocytosis was reduced by ~50% when either gene was silenced alone but ~80% when both were silenced (Figure 3B). These results suggest that, as in *C. elegans*, these genes belong to two different pathways that together account for most of the measurable phagocytosis of *E. coli* in adult mosquitoes.

We next performed epistasis experiments by using *CED6L* or *CED5L* as representatives of the two putative pathways to test whether transmembrane (*LRP1* and *BINT2*) or secreted molecules (*LRIM1*, *TEP1*, *TEP3*, and *TEP4*) belong to either pathway. Indeed, the two investigated transmembrane proteins function in different

pathways: *LRP1* in the *CED6L* pathway and *BINT2* in the *CED5L* pathway (Figures 3C and 3D). Interestingly, *LRIM1*, *TEP1*, and *TEP3* all grouped in the *CED6L* pathway (Figures 3E and 3F), whereas *TEP4* was genetically associated with *CED5L* (Figure 3F). These conclusions are summarized graphically in Figure 3G.

TEP1 and LRIM1 are both required for keeping parasite numbers low during infections of *A. gambiae* with the rodent malaria parasite *P. berghei*. It is of interest that these mosquito proteins also regulate phagocytosis of *E. coli* through *CED6L*. Additional preliminary data suggest that the subsequent components of phagocytosis are not involved in the regulation of parasite infections, as parasite development is unaffected both in *LRP1* and in *CED6L* knockdowns (S.B. and E.A.L., unpublished data).

Inactivation of Molecular Pathways Involved in Phagocytosis Causes Bacterial Accumulation in Adult *A. gambiae*

In vertebrate immunity, phagocytosis is fundamental for uptake and degradation of infectious agents and for antigen presentation (Aderem and Underhill, 1999). We reasoned that more bacteria would accumulate in mosquitoes where genes important for phagocytosis had been silenced. To test this hypothesis, we first compared the ability of *dsRNA*-treated mosquitoes to restrict bacterial growth by using *TEP1* and *DEF* as examples. Experiments consistently showed significant differences in bacterial prevalence between *dsTEP1*- and *dsGFP*-treated mosquitoes: at 8 hr after injection, approximately three times more *E. coli* (Figure 4A) and *S. aureus* (Figure 4B) were recovered from *dsTEP1* than from control *dsGFP* knockdown mosquitoes. The *dsDEF*-treated mosquitoes showed no significant enhancement in *E. coli* numbers (Figure 4C), but consistent with the strong in vivo (Blandin et al., 2002) and in vitro antigram-positive action of defensin (Vizioli et al., 2001), the levels of viable *S. aureus* were 6-fold higher in the *dsDEF* knockdown mosquitoes than in the *dsGFP* controls (Figure 4D). To test if other contributions by TEP1, other than promotion of phagocytosis, could be present, we have also compared the bacterial accumulation in *dsGFP* controls and *dsCED5L/CED6L*-treated mosquitoes. Both *E. coli* (Figure 4E) and *S. aureus* (data not shown) bacteria were recovered from *dsCED5L/CED6L*-treated mosquitoes at levels three times higher than in controls, suggesting that inhibition of phagocytosis is the reason for bacterial accumulation.

Surprisingly, the rapid initial accumulation of *E. coli* in *TEP1* knockdowns did not significantly affect subsequent mosquito survival rates over a period of 7 days (data not shown), in contrast to the known strong impact during the same period of *dsDEF* knockdown on survival of mosquitoes infected with *S. aureus* (Blandin et al., 2002). As TEP1 signals for engulfment through the CED6L pathway (Figure 3G), we wanted to exclude the possibility that the alternative CED5L pathway can compensate for this defect and mask the potential effect on mosquito survival. Therefore, we compared the survival of *E. coli*-injected *dsCED5L/CED6L* mosquitoes, which are strongly impaired in phagocytosis, with the survival of parallel single knockdowns and *dsGFP* controls; we detected no significant increase in mortality (Figure 4F).

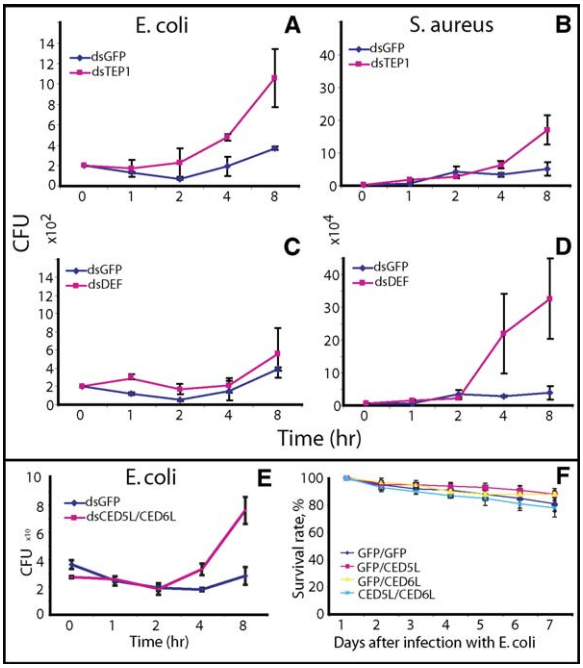

Figure 4. Analysis of Bacterial Accumulation in *TEP1*, *DEF*, and *CED5L/CED6L* Knockdown Mosquitoes after Infections with Living Bacteria

The numbers of GFP-expressing ampicillin-resistant *E. coli* (A, C, and E) and tetracycline-resistant *S. aureus* (B and D) were estimated in *dsTEP1*- (A and B), *dsDEF*- (C and D), and *dsCED5L/CED6L*- (E) injected animals and compared with numbers in *dsGFP*-injected control mosquitoes by counting colony forming units (CFUs). Bars represent the SD of the mean from three independent experiments. Simultaneous inactivation of the *CED5L* and *CED6L* engulfment pathways does not affect mosquito survival after *E. coli* injection (F).

Discussion

In this study, we have developed tools to quantitatively analyze phagocytosis in vivo in adult *A. gambiae* mosquitoes. By using this methodology in conjuction with dsRNA gene silencing, we were able to identify new regulators of phagocytosis in vivo and the dominant pathways in which they operate. We also began to explore the contribution of this process to limiting bacterial prevalence in adult mosquitoes.

Most previous screens were conducted in vitro and/or used prolonged incubation times with bacteria. This approach has some important limitations: (1) cell cultures may be deficient in circulating factors that contribute to phagocytosis; (2) no cell line is representative of all the cell types that are phagocytically active in vivo; and (3) if there is redundancy in the role of genes involved in the phagocytic process, prolonged assays may lead to a high rate of false negative results.

Learning the kinetics and studying phagocytosis at an optimal time point (30 min after bacteria injection) in adult mosquitoes facilitated the identification of numer-

ous genes involved in this process rather than in its aftermath. The fact that single knockdowns in these genes cause significant phenotypes suggests that optimal phagocytosis may depend on cooperation of different receptors activating a limited set of partially redundant pathways.

Transcriptional profiles in adult mosquitoes after septic injury (Dimopoulos et al., 2002) proved a helpful heuristic criterion in choosing genes to screen for potential roles in *E. coli* and *S. aureus* engulfment. It permitted identification of 47 genes whose inactivation changes phagocytosis of *E. coli* and/or *S. aureus* by at least 30%; 26 of these genes gave even stronger effects with one or both bacterial species and, in four cases, effects in opposite directions depending on the type of bacteria. As the vast majority of these genes had never been implicated in phagocytosis of bacteria or dying cells in other organisms, the present study constitutes a significant addition to the repertoire of factors implicated in phagocytosis. The identification of several chaperones as regulators of phagocytosis in *A. gambiae* is particularly interesting and underscores the emerging role of the ER in phagocytosis. We have found that silencing three genes that encode clear homologs of heat shock proteins (HSP70L1, HSP20L1, and HSP20L2), most likely ER resident factors, dramatically decreases the engulfment of *E. coli*. By contrast, silencing of *PPI1*, the putative ortholog of *ninaA* whose mutations in *D. melanogaster* cause ER overaccumulation (Colley et al., 1991), doubles the level of *E. coli* uptake.

Simultaneous silencing of the *CED6L* and *CED5L* genes, homologs of representative members of the two pathways governing apoptotic cell engulfment in *C. elegans*, causes a reduction of ~80% in phagocytosis of *E. coli*. The phenotype suggests that these pathways account for most if not all of the phagocytic activity in *A. gambiae*. However, the existence of a third pathway that accounts for the remaining ~20% in phagocytic activity cannot be excluded, because images collected from mosquitoes kept at +4°C after bacterial injection show no measurable fluorescence after quenching with trypan blue. In vivo analysis of epistasis experiments has provisionally assigned nine genes to these two pathways (Figure 3G), which appear to correspond to pathways implicated in phagocytosis of apoptotic cells and microorganisms in widely different species.

Silencing of genes belonging to these pathways leads to bacterial accumulation by 8 hr after infection, as would be expected from genes that affect phagocytosis. However, inactivation of the pathways, singly or in combination, does not significantly affect subsequent mosquito survival after *E. coli* infection. These data confirm previous observations in *D. melanogaster* (Elrod-Erickson et al., 2000) and suggest that, although these insect species use phagocytosis in the early antibacterial response, they rely on other, more powerful defensive tools to fight bacterial infections in the longer term. These tools presumably include bacterial killing by antimicrobial peptides. Indeed, *S. aureus* loads consistently and significantly decrease after 8 hr in the case of *dsGFP*- and *dsTEP1*-treated mosquitoes but dramatically increase in *dsDEF*-treated mosquitoes (data not shown). Additional as yet unknown effectors or other mechanisms of bacterial elimination may exist, as suggested by a dramatic decrease in the survival of *TEP4*-depleted mosquitoes after bacterial infection (S.B. and E.A.L., unpublished data). Our study has focused on two models of bacterial infection: *E. coli* and *S. aureus*. It will be of interest to test the role of phagocytosis in other infections that do not induce the production of antimicrobial peptides, as is the case for *Mycobacterium marinum* in *D. melanogaster* (Dionne et al., 2003).

In contrast to insects, phagocytosis defects in vertebrates cause profound reduction in survival of the animal after bacterial infection. One may speculate that this contrasting outcome results not only from direct pathogen elimination but also from a second distinct role of phagocytosis unique to vertebrates: antigen presentation and initiation of the adaptive response (Underhill and Ozinsky, 2002). The comparison of the repertoire of molecules used in phagocytosis in evolutionarily very distinct groups of metazoans such as insects (*A. gambiae* and *D. melanogaster*) and vertebrates (*M. musculus* and *H. sapiens*) may reveal altogether novel defense strategies as well as diversification of common pathways by the recruitment of other effectors or molecular mediators of defense reactions.

Experimental Procedures

Mosquito Colony

The *A. gambiae* strain G3 was maintained as described (Richman et al., 1996).

Double-Stranded RNA Preparation and Mosquito Injection

All cDNA templates for dsRNA preparation were verified by sequencing. Cloning details for each construct can be found in the Supplemental Data. The dsRNA was produced as previously described, and its quality was checked after annealing by gel electrophoresis; 69 nL (3 μg/μL) of dsRNA were injected in the thorax of CO_2-anaesthetized mosquito females by using a nano-injector (Nanoject, Drummond). Mosquitoes were allowed to recover for 4 days in the case of single and 5 days in the case of double knockdowns.

In Vivo Phagocytic Assay

Immobilized 5- to 7-day-old mosquitoes were injected in the thorax (see below) with 69 nL of a 1:10 dilution, from 1 mL homogeneous suspension stocks of *Escherichia coli* (K-12 strain) BioParticles, Alexa Fluor 488 conjugate, and *Staphylococcus aureus* (Wood strain without protein A). BioParticles and Alexa Fluor 594 conjugate *S. aureus* were both procured from Molecular Probes. Mosquitoes were allowed to recover and resume phagocytosis at room temperature. To quench the fluorescence of free or adherent bacteria after the desired period of time, a 138 nL trypan blue solution was injected into the same location. Mosquitoes were then partially and gently compressed between a slide and a coverslip (using clay to hold them together) for imaging. See also the Supplemental Data.

Time-Lapse Microscopy

Mosquitoes 3- to 5-days-old were immobilized on a slide positioned on ice on the universal plate of a Zeiss Axiovert microscope. The tip of a capillary needle filled with fluorescently labeled bacteria was introduced by using a fixed nanojector in the lateral side of the mosquito thorax. Image collection was initiated before injection, and frames (0.3 s exposure) were collected every second for a total period of 5 min. Very shortly after image collection was started, bacteria were injected once. Images were assembled in a video at a final speed of five frames per second.

Image Acquisition and Processing

Two nonoverlapping abdominal images were collected from each animal. Image acquisition was performed by using an inverted Zeiss Axiovert fluorescence microscope equipped with a 10× 0.3 NA Plan NEOFLUAR objective and coupled with a highly sensitive video camera (Coolsnap Fx) operated by the Metamorph software. Collected 12 bit images were analyzed with a routine written in "Interactive Data Language" (IDL, Research Systems Inc., Boulder, CO). The program includes the following steps: (1) threshold adjustment for each image by the observer, to suppress contributions not derived from internalized bacteria; (2) additional interactive masking of the remaining strongly fluorescent structures, such as autofluorescence from the wing; and (3) measurement of the total intensity of the internalized bacteria. These image-processing steps are available online in the Supplemental Data. Each experiment is the average of fluorescence measurements in five to ten mosquitoes. Experiments were repeated at least twice. In Figure 2A, no data are presented for *E. coli* after 60 min due to very high levels of diffuse fluorescence, presumably due to bacterial digestion.

Bacterial Challenge of Mosquitoes and Bacterial Survival Experiments

Bacteria were cultured at 37°C with the selective antibiotic to OD_{600} = 0.4, pelleted, washed, and resuspended in PBS to OD_{600} = 0.005 in the case of *E. coli* and OD_{600} = 0.4 in the case of *S. aureus*. Mosquitoes were anaesthetized with CO_2, injected into the thorax with 69 nL of bacterial suspension (ampicillin-resistant GFP-expressing *E. coli* from the OP50 strain or tetracycline-resistant *S. aureus* from the MSSA group [Crisostomo et al., 2001] bacteria). At the indicated time, three to five individuals were crushed, homogenized, and plated on agar plates containing the appropriate antibiotic. After overnight incubation at 37°C, the number of bacteria was estimated by counting the number of colony forming units.

Mosquito Survival

A small number of mosquitoes that were presumably damaged by the injection died within 24 hr after bacterial challenge; these were not included in the analysis. Mosquitoes that survived 24 hr but died thereafter were counted daily over a period of 7 days. Representative results of three independent experiments, each using 50 mosquitoes per tested groups, are shown.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, one figure, one table, and one movie and are available with this article online at http://www.immunity.com/cgi/content/full/23/1/65/DC1/.

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