Discovery of *Plasmodium* modulators by genome-wide analysis of circulating hemocytes in *Anopheles gambiae*

Sofia B. Pinto[a], Fabrizio Lombardo[a,1], Anastasios C. Koutsos[a,1], Robert M. Waterhouse[a], Krista McKay[b], Chunju An[b], Chandra Ramakrishnan[a], Fotis C. Kafatos[a,2], and Kristin Michel[a,b,2]

[a]Faculty of Natural Sciences, Division of Cell and Molecular Biology, Sir Alexander Fleming Building, South Kensington Campus, Imperial College London, London SW7 2AZ, United Kingdom; and [b]Division of Biology, 267 Chalmers Hall, Kansas State University, Manhattan, KS 66506-4901

Contributed by Fotis C. Kafatos, August 21, 2009 (sent for review June 20, 2009)

Insect hemocytes mediate important cellular immune responses including phagocytosis and encapsulation and also secrete immune factors such as opsonins, melanization factors, and antimicrobial peptides. However, the molecular composition of these important immune cells has not been elucidated in depth, because of their scarcity in the circulating hemolymph, their adhesion to multiple tissues and the lack of primary culture methods to produce sufficient material for a genome-wide analysis. In this study, we report a genome-wide molecular characterization of circulating hemocytes collected from the hemolymph of adult female *Anopheles gambiae* mosquitoes—the major mosquito vector of human malaria in subSaharan Africa. Their molecular profile identified 1,485 transcripts with enriched expression in these cells, and many of these genes belong to innate immune gene families. This hemocyte-specific transcriptome is compared to those of *Drosophila melanogaster* and two other mosquitoes, *Aedes aegypti* and *Armigeres subalbatus*. We report the identification of two genes as ubiquitous hemocyte markers and several others as hemocyte subpopulation markers. We assess, via an RNAi screen, the roles in development of *Plasmodium berghei* of 63 genes expressed in hemocytes and provide a molecular comparison of the transcriptome of these cells during malaria infection.

innate immunity | malaria | mosquito | cellular immunity

Hemocytes are the main mediators of the invertebrate cellular immune system but their specific functions and gene-expression profiles are poorly characterized to date. It is estimated that ≈2,000 hemocytes are present in an adult mosquito (1) but only a small fraction of these cells circulate freely in the hemolymph; most are attached to the abdominal internal organs such as the midgut and tracheae. This scarcity of circulating hemocytes has hindered their study and consequently limited the investigation of mosquito cellular immunity, in contrast with our more advanced understanding of humoral immunity.

Insect hemocytes are involved in important immune processes such as phagocytosis and encapsulation. They also contribute to humoral responses by producing antimicrobial peptides, opsonins, and components of the melanization cascade (reviewed in ref. 2). The characterization of distinct hemocyte lineages with specific functional properties in the dipteran model, *Drosophila melanogaster* (reviewed in ref. 3) has aided in the identification of hemocyte-mediated immune effector mechanisms at the molecular level. Importantly, a genome-wide transcriptional analysis of fruit fly larval hemocytes (4) identified over 2,500 genes expressed in at least one cell subpopulation, providing initial insights into the molecular repertoire of these cells.

Mosquito hemocytes have been characterized morphologically (5, 6) and based on their ability to engulf and/or encapsulate foreign objects (7, 8). In *Aedes aegypti* and *Armigeres subalbatus*, over 2,000 expressed sequence tags (ESTs) from bacteria-challenged hemocytes have been described in each species (9).

In *Anophelines*, hemocytes engulf malaria sporozoites (10) and bacteria (11) and are associated with melanotic capsules (7). Interestingly, hemocytes also express and secrete several key agonists and antagonists of malaria parasite development (6, 12–15). Three distinct morphological subpopulations of circulating hemocytes—granulocytes, oenocytoids, and prohemocytes—have been described in *Anopheles gambiae* and can be distinguished by the presence or absence of certain enzymes (6). However, none of these markers unambiguously stain a single cell population in naïve or bacteria-challenged mosquitoes, emphasizing the need to define cell population-specific markers. Pan-specific hemocyte markers, which have been instrumental for detailed analysis of cellular responses in *Drosophila*, e.g., ref. 16, remain to be identified in *An. gambiae*.

In the present study, we used a combination of transcriptional profiling, in situ hybridization, and RNA interference to analyze the transcriptional expression and immune function of circulating hemocytes in naïve and *Plasmodium berghei*-infected adult *An. gambiae* females.

Results

Hemocyte Collection and Microarray Analysis. Circulating hemocytes were collected by proboscis-clipping (17) so that virtually no contaminant tissue was detectable by microscopy. Total RNA from heads and carcasses (mosquito tissues remaining after head and circulating hemocyte removal) was also isolated. Carcass samples were used to identify potential contamination of hemocyte samples with fat body, an abundant tissue in the abdomen. Mosquito heads contain a substantial amount of neuronal tissue but few hemocytes or fat body cells. Isolated RNA was labeled via a two-cycle amplification protocol. A comparison between one-cycle and two-cycle amplified carcass RNAs showed that the extra amplification round did not introduce significant expression bias in our experimental setting (Pearson correlation coefficient of 0.908; see Fig. S1).

Identification of Hemocyte-Specific Transcripts. Strict filtering criteria were applied to identify hemocyte-specific transcripts. Only transcripts with expression values twofold above the standard deviation of global background intensities were considered. Sets of 3,959, 3,525, and 3,870 probes passed this initial filter in the

Author contributions: S.B.P., F.C.K., and K. Michel designed research; S.B.P., F.L., K. McKay, C.A., and K. Michel performed research; C.R. contributed new reagents/analytic tools; S.B.P., A.C.K., R.M.W., K. McKay, and K. Michel analyzed data; and S.B.P., F.C.K., and K. Michel wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

[1]F.L. and A.C.K. contributed equally to this work.

[2]To whom correspondence may be addressed. E-mail: f.kafatos@imperial.ac.uk or kmichel@ksu.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0909463106/DCSupplemental.

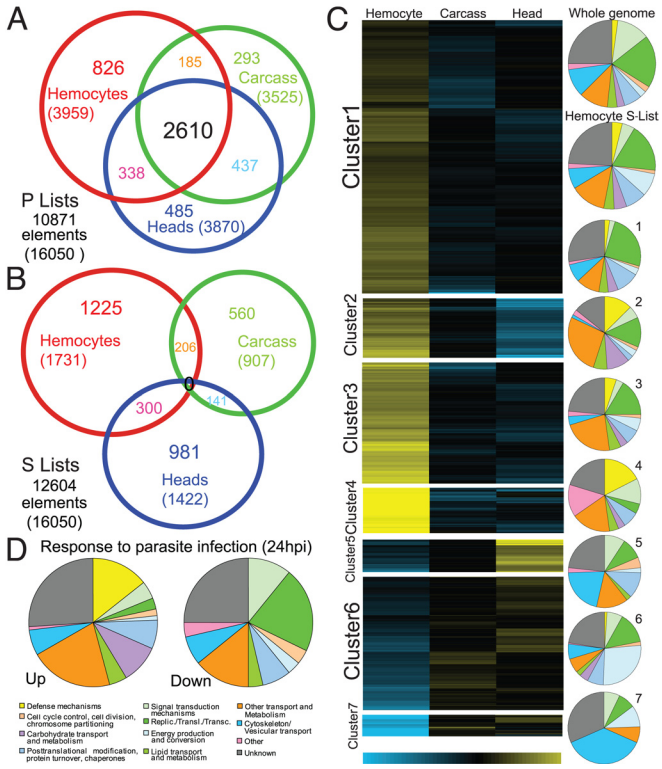

Fig. 1. Microarray profiling of circulating hemocytes. Venn diagrams representing the overlap between hemocyte (red), carcass (green), and head (dark blue) P lists (*A*) and S lists (*B*). Numbers in parentheses refer to the total numbers of elements in each list. (*C*) By using a k-means algorithm, S list genes were clustered into seven distinct coexpression clusters based on their expression intensities in the hemocyte samples. Pie charts show the relative distribution of transcripts in different molecular function groups. Yellow, high intensity; blue, low intensity; black, intensities equal to the genes used for normalization (=1). (*D*) Pie charts indicate the different molecular function groups represented by the 119 up- and 33 down-regulated transcripts after *P. berghei* infections.

hemocyte, carcass, and head samples, respectively [present (P) lists; Table S1]. These P lists were further refined, identifying 1,731, 907, and 1,422 probes, respectively, which exhibited consistent expression among the biological replicates [stringent (S) lists; Table S1; *t* test $P < 0.05$ in at least 3 of 4 hemocyte replicates]. The overlap among the three tissue P lists is 66–74% (transcripts present in all tissues; Fig. 1*A*), but no three-tissue overlap is found in the S lists with 62–71% of the probes appearing specific to each tissue (Fig. 1*B*). Although mosquito heads are almost devoid of hemocytes, carcasses likely contain many of these cells attached to various tissues. The lack of overlap between the transcript lists obtained from hemocytes and carcass samples is statistically significant (one-tailed Fisher's hypergeometric test, $P = 0.0053$), indicating that our filtering criteria identified tissue-specific gene sets.

Clustering and Annotation of Hemocyte-Enriched Transcripts. The comparative tissue analyses identified transcripts that are predominantly and potentially exclusively expressed in circulating mosquito hemocytes. K-means clustering of the 1,731 probes of the hemocyte S list partitioned the dataset into seven distinct clusters using Euclidean distance as a similarity measurement (Fig. 1*C*, Table S2). Clusters 1 to 4 contain elements with high expression (yellow) and clusters 5 to 7 contain elements with low expression in hemocytes (blue), as compared with the expression of housekeeping genes. The transcripts in each of the clusters were annotated in detail and assigned to different molecular function classes (Fig. 1*C* and Table S2 and Table S3). The distribution of molecular function groups was similar between the S list and the whole genome (Fig. 1*C*). However, several differences were visible: Clusters 2 and 4 contain a higher number of transcripts assigned to defense mechanisms indicated by a statistically significant overrepresentation of InterPro domains and gene ontology (GO) terms related to peptidases, haemocyanin and fibrinogen C domains (one-tailed Fisher's exact test with Bonferroni multiple testing correction, $P < 0.05$). Cluster 6, the largest low-expression cluster was statistically significantly enriched for GO terms related to metabolic processes and energy transduction. In total, 20 CLIP domain serine proteases, 7 SRPN, 6 LRR, 6 TEP, 5 PPO, 4 PGRP, 3 SCR, and 3 CTL transcripts are present in our S list (Table S2), all with putative functions in innate immunity (18). Also, genes previously shown to be expressed in hemocytes, such as *PP06* (19), *SP22D* (20), *CLIPB15* (14), *LYZ-C* (6), and the low level-expressed *PSMD3* (6) were detected (Table S2), lending additional credence to our approach to identifying hemocyte-specific transcripts.

Comparison of Dipteran Hemocyte Transcriptomes. The availability of published hemocyte studies from three other Dipteran species facilitated a detailed comparison with the identified *An. gambiae* hemocyte-enriched transcriptome. A microarray study in *D. melanogaster* (4) identified 2,405 transcripts as highly enriched in larval hemocytes. EST studies in the mosquitoes *Ae. aegypti* and *Ar. subalbatus* (9) established over 2,000 putative bacteria-responsive EST clusters from each species. Of these, 1,690 *Drosophila*, 604 *Aedes*, and 763 *Armigeres* genes have putative *Anopheles* 1:1 orthologs (as defined by best reciprocal BLAST hits) that are present in the Affymetrix microarray (Table 1). Only 22% of *Drosophila* genes, and 32% of the *Aedes* and 32% of *Armigeres* clusters defined in ref. 9 have putative orthologs in the *Anopheles* S list (Table 1).

This unexpected low orthology prompted further analyses of hemocyte genes in orthologous groups among *D. melanogaster*, *An. gambiae*, and *Ae. aegypti* (21). Of the 1,180 *Anopheles* genes present in the S list, 83% were in orthologous groups containing at least one *Drosophila* gene, and 90% were in orthologous groups with at least one *Aedes* gene. However, only 38% of the genes in the *Drosophila* hemocyte transcriptome were found to have orthologs in the Anopheles S list. Orthology was even less prevalent when comparing the *Anopheles* and *Aedes* hemocyte transcriptomes: Merely 30% of the Aedes hemocyte genes have orthologs in the *Anopheles* S list (Table 1). Analysis of the P list instead of the S list led to a proportional increase in the number of orthologous groups between the species. However, the percentage of orthologs detected in the respective hemocyte transcriptomes remained the same. Although the majority of identified *Anopheles* genes expressed in hemocytes have *Drosophila* and *Aedes* orthologs, their expression in hemocytes is not conserved among these dipteran species.

Microarray Validation via qRT-PCR, FISH, and IFA Analysis with Selected Genes. To validate the microarray data, real-time quantitative reverse transcription PCR (qRT-PCR) was used to measure the transcript levels of eight genes (two from each cluster 1–4) showing hemocyte-enriched expression. Results obtained from two independent biological replicates confirm the hemocyte-specific expression of the genes previously detected in the microarray assays (Fig. S3).

Additionally, 18 genes from clusters 1–4 were randomly chosen for further validation via RNA fluorescent in situ hybridizations (RNA-FISH) in ex vivo-circulating hemocytes (Table S4A, Fig. 2*A*). Two probes hybridized to all hemocyte cells tested, 14 probes labeled between 25–75% of the cells, and only two did not show any significant signal. Double in situ hybridizations showed variable degrees of expression overlap among the probes tested, and in the combinations tested, no mutually exclusive markers were found.

Peptide polyclonal antibodies were raised against the two putative pan-specific markers, AGAP007314 and AGAP002267. Im-

Table 1. Comparative analysis of dipteran hemocyte transcriptomes

Analyzed genes	Dm*	Aa†	As†	Ag‡§
1:1 Orthologs				
Number of genes	2,405	2,687	2,098	
Ag 1:1 orthologs	1,983 (1,690¶)	1,026 (604¶)	943 (763¶)	
Ag 1:1 orthologs in S list	367	191	247	
Orthologous Groups				
Total number of genes	2,756	1,461‖		1,180**
Genes in OG with Ag	2,016	1,138		1,103
Genes in OG with Dm	2,214	1,061		985
Genes in OG with Aa	2,086	1,219		1,062

Ag, *An. gambiae*; Aa, *Ae. aegypti*; As, *Ar. subalbatus*; Dm, *D. melanogaster*; OG, orthologous group defined as a set of orthologous genes from at least two species.
*Hemocyte transcriptomes from ref. 4
†Hemocyte transcriptomes from ref. 9.
‡S list.
§ The Ag genes with an ortholog in the *Dm* hemocyte set (i.e., hemocyte transcriptomes from ref. 4) numbered 369. The Ag genes with an ortholog in the Aa hemocyte set (i.e., hemocyte transcriptomes from ref. 9) numbered 319.
¶Numbers in parentheses represent the number of those *An. gambiae* orthologs that are present in the *Anopheles* Affymetrix array.
‖Number of *Aedes* genes mapping to the set of hemocyte-enriched ESTs.
**Number of *Anopheles* genes mapping to the set of hemocyte-enriched Affymetrix probes.

munofluorescence analysis (IFA) confirmed their protein presence in 93.3% and 98.5% of circulating hemocytes, respectively. The presence of two additional proteins, AGAP11765 and AGAP09231, was confirmed in a subpopulation of circulating hemocytes (Fig. 2*B*, Table S4B). Moreover, available antibodies whose transcripts were detected in this study as hemocyte products, were tested by IFA. Anti-PPO2, -CLIPA8, -CLIPB8, -CLIPB17 and -LRIM1 antibodies stained perfused hemocytes, confirming their expression in the circulating hemocyte population (Fig. 2*B*). CLIPB4 was not detected in the microarray analysis and IFAs labeled only one of >50 cells.

Taken together, hemocyte-enriched expression was confirmed independently for 29 of 30 genes tested, strongly supporting our microarray results.

RNAi Screen to Identify New *Plasmodium* Immune Modulators. We chose a set of 63 putative immune modulators to be tested for their effect on *P. berghei* early sporogonic development in gene knockdown (KD) experiments based on several filtering criteria listed in Table S5: (*i*) high expression levels in hemocytes, (*ii*) differential expression after parasite infection, determined from all available expression data in Vectorbase (www.vectorbase.org), (*iii*) presence or absence of signal peptide and transmembrane domain(s), (*iv*)

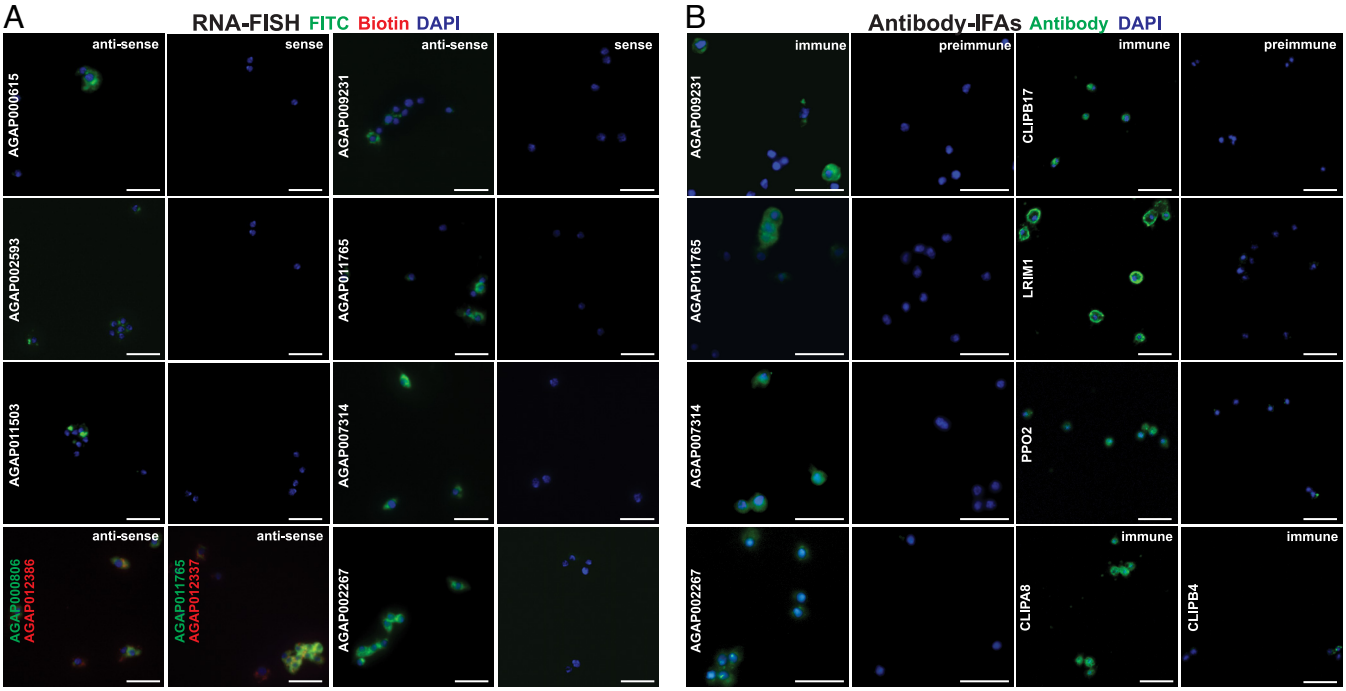

Fig. 2. Validation of naïve hemocyte microarrays. (*A*) Microarray results were validated by FISH, probes used in single-labeled hybridizations are shown in white, and those used in double-labeling experiments are indicated in green and red. (*B*) FISH probes were further confirmed by antibody immunofluorescence. (*C*) In addition available antibodies to identified markers were also tested. Scale bars: 10 μm.

Table 2. Summary of RNAi screen candidates

Gene KD	N	Developing Oocysts						Melanized Ookinetes				
		Md	Load*	Fd	$P^{\dagger}$	Pr (%)‡	$\chi^{2\S}$	Load*	Fd	$P^{\dagger}$	Pr (%)‡	$\chi^{2\S}$
Genes affecting oocyst load and prevalence of infection												
AGAP004016¶	35	2	14 ± 3.9	0.4	**0.004**	63	**1e^{-4}**	11 ± 0.7	9.4	0.064	42	**0.007**
LacZ		16	35 ± 7.1			86		1 ± 5.3			20	
AGAP006914¶	47	5	27 ± 6.4	0.6	**0.041**	74	**0.009**	6 ± 2.8	1.4	0.307	45	**0.009**
LacZ		19	41 ± 7.9			83		5 ± 2.1			28	
AGAP010325¶	41	13	28 ± 5.5	2.2	**0.017**	85	**0.008**	1 ± 0.4	0.3	0.212	20	**0.048**
LacZ		6	13 ± 3.5			66		3 ± 1.4			34	
EST08518¶	47	2	13 ± 3.9	0.4	**0.002**	57	**4e^{-4}**	13 ± 10	8.6	0.641	30	0.500
LacZ		19	36 ± 7.5			79		2 ± 0.6			26	
Genes affecting oocyst load only												
AGAP009642	54	24	49 ± 8.4	1.8	**0.017**	89	0.465	4 ± 1.4	2.2	0.341	32	0.233
LacZ		11	28 ± 6.6			86		2 ± 0.7			25	
AGAP005761	45	15	43 ± 10	2.3	**0.047**	82	0.177	8 ± 4.9	2.9	0.540	22	0.197
LacZ		6	19 ± 5.2			77		3 ± 1.3			31	
Genes affecting ookinete melanization load and prevalence of ookinete melanization												
AGAP003304¶	52	11	40 ± 8.2	1.0	0.252	83	0.192	11 ± 3.7	2.5	**0.021**	54	**3e^{-4}**
LacZ		17	39 ± 7.4			88		4 ± 1.8			31	
AGAP012000¶	55	17	38 ± 6.9	0.9	0.441	82	0.715	17 ± 6.9	5.8	**0.021**	45	**2e^{-6}**
LacZ		21	41 ± 7.4			84		3 ± 1.5			20	
AGAP001508¶	42	7	30 ± 9.6	0.8	0.273	69	1.0	9 ± 5.1	1.5	**0.040**	77	**7e^{-8}**
LacZ		16	38 ± 10			69		6 ± 4.7			17	
Genes affecting prevalence of ookinete melanization												
AGAP007314¶	32	2	14 ± 3.7	0.8	0.460	62	0.710	17 ± 8.6	3.2	0.118	53	**0.008**
LacZ		2	18 ± 8.1			66		5 ± 2.3			31	
AGAP001242¶	48	30	57 ± 9.4	1.7	0.157	77	0.220	6 ± 3.2	6.7	0.144	31	**0.007**
LacZ		20	34 ± 7.2			85		1 ± 0.5			16	
AGAP009380¶	57	6	13 ± 3.2	1.2	0.674	70	0.547	2 ± 0.8	0.2	0.099	17	**0.011**
LacZ		7	11 ± 1.9			66		6 ± 2.3			33	

All significant *P* values (<0.05) and chi-square test values are highlighted in bold. KD, knocked down; N, number of mosquitoes tested per KD; Md, median; Fd, fold difference; Pr, prevalence.

*Load represents the arithmetic mean (+/− one Standard Error of the Mean) of parasite numbers, including uninfected guts, in three biological repeats.

†Mann–Whitney U test on oocyst or melanized ookinete load.

‡Percent sign refers to the percentage of infected mosquitoes.

§Chi-square test on oocyst or melanized ookinete prevalence.

¶All gene KDs with an effect on prevalence of infection and/or melanization were further analyzed to determine if parasite load is affected once prevalence effect is eliminated from the analysis by removing uninfected midguts from the dataset. Two gene KDs showed an additional effect of infection intensity: *AGAP004016* (N = 22, dsGOI: 23 ± 5.5, dsLacZ: 61 ± 17, P = 0.02) and *ENSANGESTG00000008518* (N = 27, dsGOI: 20 ± 6.2, dsLacZ: 50 ± 12; P = 0.007).

presence of Interpro domains associates with immune functions, (*v*) absence of any annotated protein domain, and (*vi*) orthology to transcripts present in other dipteran hemocyte transcriptomes. All 63 candidates were assayed once, and parasite numbers were compared with control pools. KD was repeated a second time for 39 candidates, for which KD either (*i*) decreased by at least 50% or increased by at least 70% parasite load, or (*ii*) had a considerable effect on infection prevalence. Following these criteria, 20 gene KDs were repeated a third time. Screen data are shown in Table S5, and statistically significant results are summarized in Table 2.

KD of *AGAP004016*, *AGAP006914*, *and ENSANGESTG00000008518* resulted in a significant reduction of oocyst load and infection prevalence. *AGAP004016*-KD and *AGAP006914*-KD also led to a significant increase in parasite melanization prevalence. Reduction of transcript levels of *AGAP005761*, *AGAP009642*, and *AGAP010325* caused an increase in oocyst load, but only *AGAP010325*-KD also significantly increased the prevalence of oocyst infection.

A few gene KDs affected the presence of melanized ookinetes in the susceptible G3 strain of mosquitoes without affecting oocyst load. KD of *AGAP003304*, *AGAP012000*, or *AGAP001508* significantly increased melanized ookinete load as well as the prevalence of melanization. *DsAGAP007314* and *dsAGAP001242*-injected mosquitoes increased, whereas *dsAGAP009380* significantly decreased prevalence of melanization; none of these genes affected melanized ookinete load. KD of transcript levels was confirmed in whole mosquitoes by qRT-PCR for three of these genes (Fig. S2).

Microarray Analysis of Hemocytes Isolated from *P. berghei*-Infected *An. gambiae*. In a second set of microarray experiments, global expressional changes after infection with malaria parasites were assessed in *An. gambiae* hemocytes. Infections were performed by using the infectious EGFP-CON *P. berghei* strain (22) or an isogenic, but invasion-deficient, *C*ircumsporozoite- and *T*RAP-*r*elated *p*rotein (CTRP) knockout strain (CTRPko/GFP). Hemocytes from the CTRPko/GFP-infected mosquitoes served as reference and should reveal transcriptional changes specifically associated with parasite invasion. The time point for analysis was chosen to coincide with the ookinete reaching the basal lamina of the midgut epithelium (24–28 h), where the parasite is potentially interacting with factors released into the hemolymph by hemocytes. In total, 162 probes mapping to 142 transcripts were found to be significantly changed by infection (one-way ANOVA, $P < 0.05$; Table S6): 33 transcripts were at least twofold lower expressed in the infected vs. control samples, whereas 119 transcripts had at least twofold higher signals (Fig. 1*D*). Increased transcript levels of several genes were confirmed independently by qRT-PCR (Fig. S3*B*).

Of the 142 regulated transcripts, 43%, 12%, and 16% are present in the uninfected hemocyte, carcass, and head S lists (Table S6).

IMMUNOLOGY

Surprisingly, the 11 genes with the highest transcript level changes due to infection were present in the carcass S list, suggesting potential infection-dependent recruitment of normally attached hemocytes into circulation. The 119 up-regulated transcripts belong to a variety of functional classes (Fig. 1*D*), including metabolism and transport of carbohydrates and other molecules. It is noteworthy that 13% of transcripts encode secretory proteins with putative immunity function, whereas this functional class only makes up 2% of the whole transcriptome of *An. gambiae* (Fig. 1*D*). No immune-related genes were found to be down-regulated by infection. Of the 12 genes that have been tested by RNAi for an effect on malaria parasite survival, seven have shown a significant effect on either oocyst load or ookinete melanization (Table S6), revealing a significant contribution of circulating hemocytes to the humoral immune repertoire of the mosquito.

Discussion

Cellular immune responses are central to innate immunity in any invertebrate. Specialized blood cells capable of recognizing foreign intruders directly eliminate them by engulfment or encapsulation or to signal other tissues to activate systemic or local responses (23). Very little is known of the molecular mechanisms underlying cellular immune responses in mosquitoes despite the fact that many of the molecules modulating development of the malaria parasite are expressed by these cells (12, 14, 15).

By using microarray analyses, we have compiled a list of 1,485 transcripts (S list, Table S1) that are expressed in hemocytes. These transcripts were grouped into seven coexpression clusters, which include 1,053 genes that are predominantly expressed in hemocytes of adult female *An. gambiae* (Clusters 1–4). A parallel study by Baton et al. (24) recently identified 174 genes specifically expressed in adult hemocytes, 35 and 14 of which we were able to detect in our microarray study as hemocyte- and carcass-specific, respectively. Interestingly, clusters 2–4 contained a higher percentage of genes involved in defense mechanisms, in comparison with the whole genome, as reflected by the significant overrepresentation of peptidase domains and the presence of a substantial number of annotated immunity genes (18). Clusters 5–7 showed low hemocyte expression. Interestingly, cluster 6 showed significant enrichment for GO terms for metabolic and energy-transducing processes as well as annotations for posttranslational modifications and energy production. These transcripts are likely to represent genes required for energy household and general metabolism and are probably expressed in many mosquito tissues, especially the fat body.

Surprisingly, although the genes expressed in *An. gambiae* hemocytes are evolutionarily well conserved among other dipteran species (*D. melanogaster*, *Ae. aegypti*, *Ar. subalbatus*), their expression in hemocytes is often not preserved. The different experimental designs (different developmental stages, physiological states, analysis criteria) that were used in the individual studies have likely contributed to this disparity. However, the need to respond to the different immune challenges encountered in the natural environments of these different dipterans could have shaped differences in the molecular repertoires of these cells.

The *Anopheles* hemocyte transcript catalog we have generated was used to identify molecular markers. Two proteins with no assigned function were identified as pan-specific markers for circulating hemocytes. Fourteen other genes were expressed in overlapping but distinct subsets of circulating hemocytes, indicating a complex and plastic population structure among these cells.

Previous work had suggested that hemocytes contribute significantly to the immune defense against *Plasmodium sp*. (12). We took a systematic approach to further elucidate the mechanisms by which as subset of hemocyte-derived factors affect parasite survival in the mosquito. Twelve modulators of *P. berghei* development, approximately one-fifth of the 63 genes assayed, were identified. Some of these factors contain functional domains found within proteins that have been shown previously to affect parasite development, e.g., leucine-rich repeats (25), Ig-like domains (26), and Fibrinogen C domains (27). The contribution of these functional domains to antiparasite immunity is diverse, and each is likely to affect several pathways. Additionally, we identified several novel hemocyte factors (ENSANGESTG00000008518, AGAP001508, AGAP005761, AGAP003304) as modulators of parasite infection. Future functional studies will assess specific molecular functions and their roles within innate immune pathways in *An. gambiae*.

In addition to constitutively producing factors that affect parasite development, circulating hemocytes change their transcriptional profiles upon ookinete traversal of the midgut epithelium. Of the 119 transcripts with increased hemocyte expression levels that we have identified, 16 encode known immunity-related proteins (28), and six of those have already been shown to have direct or indirect antiparasite activity (29–31). This suggests that the increased expression of a hemocyte transcript during infection is a good indicator of its potential role in modulating parasite development.

In summary, this study provides an extensive catalog of hemocyte-enriched molecules and identifies two pan-specific markers for circulating hemocytes in *An. gambiae*. The data further support the hypothesis that mosquito circulating hemocytes contribute substantially to the immune responses against malaria parasites.

Materials and Methods

Mosquito Rearing and *P. berghei* Infections. An *An. gambiae* G3 strain was maintained according to ref. 32. The *P. berghei* GFP-CON 259cl2 strain (22) and the *P. berghei* CTRPko/GFP strain were passaged in WT female mice; infections were performed as described (33). Oocyst prevalence and load were determined in dissected midguts (8–10 dpi) by fluorescence microscopy.

RNA Isolation. Hemocytes were harvested from 500 infected or 1,000 naive females by proboscis clipping. RNA was extracted according to the RNeasy Mini protocol for animal cells (Qiagen). Twenty carcasses (mosquito tissues after hemocyte collection and decapitation), and 40 heads were collected into TRIzol Reagent (Invitrogen) and RNA extracted according to manufacturer's protocol. Before RNA labeling, TRIzol-isolated RNA was cleaned by MiniRNeasy column (Qiagen) according to the manufacturer's instructions.

RNA Labeling and Microarray Hybridization. Total RNA was biotinylated according to the one-cycle (only carcass samples) or the two-cycle eukaryotic target labeling protocol (hemocytes, carcass and head samples) (Affymetrix) by using 1 μg and 80 ng as a starting amount, respectively. All labeled cRNAs were cleaned, fragmented, hybridized to GeneChip *Plasmodium/Anopheles* genome arrays (Affymetrix) and scanned following manufacturer's instructions.

Microarray Data Analysis. Analysis of the microarrays was performed by using the GCOS 1.4 (Affymetrix) and GeneSpring GX 7.3 software (Agilent Technologies). In naïve experiments, raw data were normalized by median normalization to a set of 65 ribosomal housekeeping genes of *An. gambiae* identified by ref. 34. For filtering criteria see *Results* section. In infection experiments, data were normalized (*i*) per chip to 50th percentile and (*ii*) per gene to median. Signals were considered present when flagged present in 2 of 6 experiments using GCOS flags. Expression levels between hemocytes from infected and control-fed mosquitoes were considered significantly different when at or above a fold change of two- and a one-way ANOVA of $P < 0.05$.

Microarray data sets have been submitted to GEO: GSE17919 (naïve experiments) and GSE17866 (infection experiments).

1:1 Ortholog Comparison. BLAST searches of a list of 2,405 hemocyte-enriched *D. melanogaster* genes from ref. 4 were performed against the *An. gambiae* genome. 1:1 orthologs were defined by best reciprocal BLAST hits. Similarly, BLAST searches of 2,686 *Ae. aegypti* and 2,098 *Ar. subalbatus* hemocyte sequences from EST libraries (9) identified putative 1:1 orthologs in *An. gambiae*.

Orthologous Group Comparison. Orthologous group delineation was carried out through a four-species comparison among the predicted proteomes of *D. melanogaster*, *Ae. aegypti*, and *An. gambiae*, with *Apis mellifera* as an outgroup (21). The 2,756 *Drosophila* FlyBase gene identifiers with more than twofold expression difference between the transcriptomes of unchallenged hemocytes and whole larvae were extracted from Irving et al. (4). The 2,686 *Ae. aegypti* hemocyte EST clusters (9) were mapped to the full set of predicted *Aedes* transcripts at Vector-

base (35) by using BLAT [FastMap (36)], requiring a match length of ≥50 bp and >20% of the EST or transcript length.

Single-Stranded RNA Probes for FISH. Target cDNAs were cloned by using primers present in Table S5, into pGemT-easy vector (Promega). Roughly 1–2 μg of plasmid DNA was linearized in two separate reactions with restriction enzymes NdeI and NcoI. Sense and antisense probes were synthesized by nick translation following manufactures instructions (Roche).

RNA FISH in ex Vivo Hemocytes. Circulating hemocytes were collected by proboscis clipping into 2 μL of Schneider medium (Gibco) and fixed in 4% formaldehyde for 10min. Endogenous HRP was quenched with 1% H_2O_2 in phosphate-buffered saline (PBS) with 0.1% Triton X (PBT) and washed twice. Hemocytes were prehybridized in 50% formamide, 5× saline sodiumcitrate (SSC) buffer, 100 mg/mL heparin, 100 mg/mL sonicated Herring sperm DNA, 0.1% Tween-20 at 56° C for 2 h followed by probe hybridization (100 ng) at 56° C overnight. Cells were washed at 56° C 3× in 50% formamide, 5× SSC, 0.1% Tween-20, and twice with PBT before cooling to room temperature (RT). Probe detection was performed at RT with α-fluorescein (FITC)-conj-HRP Ab (1/500, Molecular Probes) and/or SA-HRP (1/200, PerkinElmer) in 1× PBS, 0.1% Triton X, 1% milk (PBTB) for 1.5 h. Samples were washed once in PBTB and twice in PBT. Tyramide signaling amplification development followed manufacturer's instructions (PerkinElmer). Samples were counterstained with DAPI (2 ng/mL, Roche), mounted in VectaShield (Vector Laboratories) and analyzed by using an Axiovert fluorescence microscope (Zeiss).

Antibody Production. Predicted sequences of selected candidates were validated from G3 females cDNA by cloning full-length sequences into the pIEX expression system by using primers of Table S5. Recombinant His-tagged proteins were expressed in *Sua5.1** mosquito cell lines and correctly detected with an a-His antibody in western analysis. The appropriate candidate epitopes for immunization (Table S5) were selected according to the protocols and procedures of Eurogentec.

IFA. Circulating hemocytes were prepared for IFA as described in ref. 15 and incubated with the antibodies listed in the *SI Text*. When available, preimmune serum was used at the same dilution as the respective antisera. Counterstaining, mounting, and analysis were performed as described in *RNA FISH in ex Vivo Hemocytes*.

***P. berghei* Infection Screen via Double-Stranded RNA KD.** Candidate genes were PCR amplified by T7-tailed primers (Table S5), and dsRNAs were synthesized according to ref. 37. Injections were performed as previously described (15).

Candidate genes were selected based on high expression in hemocytes and their annotations [e.g., protein domains, differential expression in previous immune studies (38, 39)]. Results were statistically analyzed as previously described (15).

ACKNOWLEDGMENTS. We thank European Molecular Biological Laboratory GeneCore Facility and L. Steinmetz for assistance with microarray experiments, and the Bioinformatics Center and Center of Biomedical Research Excellence Molecular and Biochemistry Core at Kansas State University for their support. Thanks also go to M. A. Osta (American University of Beirut, Lebanon), H. M. Mueller (University of Heidelberg, Germany), A. K. Schnitger (Imperial College London), and J. Volz (The Walter and Eliza Hall Institute of Medical Research, Melbourne) for sharing antibodies and to D. Doherty (EMBL, Heidelberg, Germany), T. Habtewold (Imperial College London), K. Kjos and C. Paddock (Kansas State University, Manhattan, KS) for mosquito rearing. This work was supported by FP6 BioMalPar Grant E LSHP-CT-2004–503578, Wellcome Trust Grant PG GR077229MA, and National Institutes of Health/National Institute of Allergy and Infectious Diseases Grants P01 AI044220–06AI and P20 RR016475, and NIH-P20-RR017686-Core. R. M. W. was supported by a Wellcome Trust PhD fellowship.

1. Christensen BM, Huff BM, Miranpuri GS, Harris KL, Christensen LA (1989) Hemocyte population changes during the immune response of Aedes aegypti to inoculated microfilariae of Dirofilaria immitis. *J Parasitol* 75:119–123.
2. Lavine MD, Strand MR (2002) Insect hemocytes and their role in immunity. *Insect Biochem Mol Biol* 32:1295–1309.
3. Meister M (2004) Blood cells of Drosophila: Cell lineages and role in host defence. *Curr Opin Immunol* 16:10–15.
4. Irving P et al. (2005) New insights into Drosophila larval haemocyte functions through genome-wide analysis. *Cell Microbiol* 7:335–350.
5. Hillyer JF, Christensen BM (2002) Characterization of hemocytes from the yellow fever mosquito, *Aedes aegypti*. *Histochem Cell Biol* 117:431–440.
6. Castillo JC, Robertson AE, Strand MR (2006) Characterization of hemocytes from the mosquitoes *Anopheles gambiae* and *Aedes aegypti*. *Insect Biochem Mol Biol* 36:891–903.
7. Hernandez–Martinez S, Lanz H, Rodriguez MH, Gonzalez–Ceron L, Tsutsumi V (2002) Cellular-mediated reactions to foreign organisms inoculated into the hemocoel of *Anopheles albimanus* (Diptera: Culicidae). *J Med Entomol* 39:61–69.
8. Hillyer JF, Schmidt SL, Christensen BM (2003) Rapid phagocytosis and melanization of bacteria and Plasmodium sporozoites by hemocytes of the mosquito *Aedes aegypti*. *J Parasitol* 89:62–69.
9. Bartholomay LC, et al. (2004) Description of the transcriptomes of immune response-activated hemocytes from the mosquito vectors *Aedes aegypti* and *Armigeres subalbatus*. *Infect Immun* 72:4114–4126.
10. Hillyer JF, Barreau C, Vernick KD (2007) Efficiency of salivary gland invasion by malaria sporozoites is controlled by rapid sporozoite destruction in the mosquito haemocoel. *Int J Parasitol* 37:673–681.
11. Levashina EA, et al. (2001) Conserved role of a complement-like protein in phagocytosis revealed by dsRNA knockout in cultured cells of the mosquito, *Anopheles gambiae*. *Cell* 104:709–718.
12. Blandin S, et al. (2004) Complement-like protein TEP1 is a determinant of vectorial capacity in the malaria vector *Anopheles gambiae*. *Cell* 116:661–670.
13. Frolet C, Thoma M, Blandin S, Hoffmann JA, Levashina EA (2006) Boosting NF-kappaB-dependent basal immunity of *Anopheles gambiae* aborts development of Plasmodium berghei. *Immunity* 25:677–685.
14. Volz J, Osta MA, Kafatos FC, Muller HM (2005) The roles of two clip domain serine proteases in innate immune responses of the malaria vector *Anopheles gambiae*. *J Biol Chem* 280:40161–40168.
15. Abraham EG, et al. (2005) An immune-responsive serpin, SRPN6, mediates mosquito defense against malaria parasites. *Proc Natl Acad Sci USA* 102:16327–16332.
16. Goto A, et al. (2001) A Drosophila haemocyte-specific protein, hemolectin, similar to human von Willebrand factor. *Biochem J* 359:99–108.
17. Chen CC, Laurence BR (1987) In vitro study on humoral encapsulation of microfilariae: Establishment of technique and description of reactions. *Int J Parasitol* 17:781–787.
18. Christophides GK, et al. (2002) Immunity-related genes and gene families in Anopheles gambiae. *Science* 298:159–165.
19. Muller HM, Dimopoulos G, Blass C, Kafatos FC (1999) A hemocyte-like cell line established from the malaria vector *Anopheles gambiae* expresses six prophenoloxidase genes. *J Biol Chem* 274:11727–11735.
20. Danielli A, et al. (2000) A modular chitin-binding protease associated with hemocytes and hemolymph in the mosquito Anopheles gambiae. *Proc Natl Acad Sci USA* 97:7136–7141.
21. Kriventseva EV, Rahman N, Espinosa O, Zdobnov EM (2008) OrthoDB: The hierarchical catalog of eukaryotic orthologs. *Nucleic Acids Res* 36:D271–D275.
22. Franke–Fayard B, et al. (2004) A *Plasmodium berghei* reference line that constitutively expresses GFP at a high level throughout the complete life cycle. *Mol Biochem Parasitol* 137:23–33.
23. Lemaitre B, Hoffmann J (2007) The host defense of *Drosophila melanogaster*. *Annu Rev Immunol* 25:697–743.
24. Baton LA, Robertson A, Warr E, Strand MR, Dimopoulos G (2009) Genome-wide transcriptomic profiling of *Anopheles gambiae* hemocytes reveals pathogen-specific signatures upon bacterial challenge and *Plasmodium berghei* infection. *BMC Genomics* 10:257.
25. Osta MA, Christophides GK, Kafatos FC (2004) Effects of mosquito genes on Plasmodium development. *Science* 303:2030–2032.
26. Garver LS, Xi Z, Dimopoulos G (2008) Immunoglobulin superfamily members play an important role in the mosquito immune system. *Dev Comp Immunol* 32:519–531.
27. Dong Y, Dimopoulos G (2009) Anopheles fibrinogen-related proteins provide expanded pattern recognition capacity against bacteria and malaria parasites. *J Biol Chem* 284:9835–9844.
28. Waterhouse RM, et al. (2007) Evolutionary dynamics of immune-related genes and pathways in disease-vector mosquitoes. *Science* 316:1738–1743.
29. Kim W, et al. (2004) Ectopic expression of a cecropin transgene in the human malaria vector mosquito *Anopheles gambiae* (Diptera: Culicidae): Effects on susceptibility to *Plasmodium*. *J Med Entomol* 41:447–455.
30. Michel K, Budd A, Pinto S, Gibson TJ, Kafatos FC (2005) *Anopheles gambiae* SRPN2 facilitates midgut invasion by the malaria parasite *Plasmodium berghei*. *EMBO Rep* 6:891–897.
31. Volz J, Muller HM, Zdanowicz A, Kafatos FC, Osta MA (2006) A genetic module regulates the melanization response of Anopheles to Plasmodium. *Cell Microbiol* 8:1392–1405.
32. Richman AM, et al. (1996) Inducible immune factors of the vector mosquito *Anopheles gambiae*: Biochemical purification of a defensin antibacterial peptide and molecular cloning of preprodefensin cDNA. *Insect Mol Biol* 5:203–210.
33. Sinden RE (1996) Infection of mosquitoes with rodent malaria. In *Molecular Biology of Insect Disease Vectors*: *A Methods Manual*, eds. Crampton JM, Beard CB, Louis C (Chapman & Hall, London), pp. 67–91.
34. Marinotti O, et al. (2006) Genome-wide analysis of gene expression in adult *Anopheles gambiae*. *Insect Mol Biol* 15:1–12.
35. Lawson D, et al. (2007) VectorBase: A home for invertebrate vectors of human pathogens. *Nucleic Acids Res* 35:D503–D505.
36. Kent WJ (2002) BLAT–The BLAST-like alignment tool. *Genome Res* 12:656–664.
37. Meister S, et al. (2005) Immune signaling pathways regulating bacterial and malaria parasite infection of the mosquito *Anopheles gambiae*. *Proc Natl Acad Sci USA* 102:11420–11425.
38. Dong Y, et al. (2006) *Anopheles gambiae* immune responses to human and rodent Plasmodium parasite species. *PLoS Pathog* 2:e52.
39. Vlachou D, Schlegelmilch T, Christophides GK, Kafatos FC (2005) Functional genomic analysis of midgut epithelial responses in *Anopheles* during *Plasmodium* invasion. *Curr Biol* 15:1–11.