**Anopheles gambiae** Antiviral Immune Response to Systemic O’nyong-nyong Infection

Joanna Waldock¹, Kenneth E. Olson², George K. Christophides¹*

1 Division of Cell and Molecular Biology, Department of Life Sciences, Imperial College, London, United Kingdom, ²Arthropod Infectious Diseases Laboratory, Colorado State University, Fort Collins, Colorado, United States of America

Abstract

**Background:** Mosquito-borne viral diseases cause significant burden in much of the developing world. Although host-virus interactions have been studied extensively in the vertebrate host, little is known about mosquito responses to viral infection. In contrast to mosquitoes of the Aedes and Culex genera, *Anopheles gambiae*, the principal vector of human malaria, naturally transmits very few arboviruses, the most important of which is O’nyong-nyong virus (ONNV). Here we have investigated the *A. gambiae* immune response to systemic ONNV infection using forward and reverse genetic approaches.

**Methodology/Principal Findings:** We have used DNA microarrays to profile the transcriptional response of *A. gambiae* inoculated with ONNV and investigate the antiviral function of candidate genes through RNAi gene silencing assays. Our results demonstrate that *A. gambiae* responses to systemic viral infection involve genes covering all aspects of innate immunity including pathogen recognition, modulation of immune signalling, complement-mediated lysis/opsonisation and other immune effector mechanisms. Patterns of transcriptional regulation and co-infections of *A. gambiae* with ONNV and the rodent malaria parasite *Plasmodium berghei* suggest that hemolymph immune responses to viral infection are diverted away from melanisation. We show that four viral responsive genes encoding two putative recognition receptors, a galectin and an MD2-like receptor, and two effector lysozymes, function in limiting viral load.

**Conclusions/Significance:** This study is the first step in elucidating the antiviral mechanisms of *A. gambiae* mosquitoes, and has revealed interesting differences between *A. gambiae* and other invertebrates. Our data suggest that mechanisms employed by *A. gambiae* are distinct from described invertebrate antiviral immunity to date, and involve the complement-like branch of the humoral immune response, supressing the melanisation response that is prominent in anti-parasitic immunity. The antiviral immune response in *A. gambiae* is thus composed of some key conserved mechanisms to target viral infection such as RNAi but includes other diverse and possibly species-specific mechanisms.

Introduction

Arthropod-borne viruses (arboviruses) are a significant health burden across the world. They represent an emerging and resurgent group of pathogens [1], many of which are transmitted by mosquitoes including Dengue Fever (DEN), Yellow Fever (YF), West Nile Virus (WNV) and Chikungunya (CHIKV). The development of control approaches. Mosquitoes launch robust immune responses against a variety of pathogens: recognition of pathogen associated molecular patterns (PAMPS) leads to activation of immune signalling pathways associated with production of potent anti-microbial peptides (AMPs) or cascades that lead to pathogen lysis, phagocytosis, melanisation or cellular encapsulation by hemocytes, the white only recently that invertebrate antiviral immunity has received analogous attention. Initial studies have used *Drosophila melanogaster* as a model system, as the power of genetics and the extensive knowledgebase in *Drosophila* have been invaluable in establishing the foundations for insect antiviral immunity research. However, the biology of arboviruses is tightly linked to the physiology of haematophagous arthropods, and as such research in model organisms may not be fully relevant to the transmission of viruses and associated vector defence. A forward research approach is required to effectively study the vector responses to arboviruses, utilizing findings in *Drosophila* as guidance.

Mosquitoes launch robust immune responses against a variety of pathogens: recognition of pathogen associated molecular patterns (PAMPS) leads to activation of immune signalling pathways associated with production of potent anti-microbial peptides (AMPs) or cascades that lead to pathogen lysis, phagocytosis, melanisation or cellular encapsulation by hemocytes, the white
blood cell equivalents [2]. To date three signalling pathways have been implicated in mosquito antiviral immunity. The JAK/STAT pathway, a known antiviral signalling pathway in mammals [3], appears to have a conserved function in Aedes aegypti. JAK/STAT related genes are differentially regulated in response to DENV infection [4], and Aedes aegypti can be made more or less susceptible to DENV through silencing of DOME (a receptor of the JAK/STAT pathway) and PIAS (a negative regulator of the JAK/STAT pathway) respectively [5]. In addition, 18 genes downstream of the Ae. aegypti JAK/STAT pathway are regulated by DENV infection, two of which have been shown to be DENV antagonists [5]. The RNAi pathway has been demonstrated to limit viral infection in several mosquito vector-virus combinations. AgAGO2 (a member of the RISC complex) is an antagonist of ONNV in A. gambiae [6]; AeAGO2, AeDCR2 and AeTTSV (all members of the RNAi pathway) are Sindbis virus (SINV) antagonists in Ae. aegypti [7]; AeDCR2 had also been shown to be a DENV antagonist [8]. The presence of viRNA (siRNA that is specific to viral genomes) has been demonstrated in Ae. aegypti infected with SINV and DENV [9,10], and recombinant viruses encoding suppressors of RNAi have been shown to increase mortality, increase viral titres and lower the build-up of viRNAs in infected mosquitoes [9,10]. Finally, Toll pathway related genes are differentially regulated in response to both SINV and DENV infection in Ae. aegypti [4,11]. Activation and inhibition of the Toll pathway has been demonstrated to respectively decrease and increase susceptibility to different DENV strains in different Ae. aegypti strains showing the importance of the Toll pathway in mosquito antiviral immunity [4,12].

Whereas the Aedes and Culex mosquitoes transmit numerous viruses, Anopheles mosquitoes (the principal vectors of malaria) are known to be the primary vectors of only O’nyong-nyong virus (ONNV). ONNV is a positive (sense) strand single stranded RNA (+ssRNA) virus of the Alphavirus family, with reported epidemics in West Africa in the 1960s and 1990s [13–18]. Viral replication of ONNV in A. gambiae is shown to be slow and restricted in tissue tropism compared to most vector-virus combinations [19]. Permissiveness to infection has been shown, in part, to be regulated by RNAi, and inhibition of RNAi results in high susceptibility to viral infection [6]. Here we have profiled the global transcriptional responses of A. gambiae to ONNV infection of the hemolymph to identify viral responsive genes, and then used RNAi silencing to test a selection of identified genes for antiviral function. Our results confirm that in A. gambiae the RNAi pathway is a key antiviral mechanism, however, the JAK/STAT and the Toll pathway do not have a significant role in regulating systemic ONNV infection. We further identify four viral responsive genes with novel functions in mosquito antiviral immunity. Patterns of immune gene expression coupled with co-infections of A. gambiae with the rodent malaria parasite P. berghei suggest that viral infection inhibits parasite melanisation. Overall, we demonstrate that A. gambiae uses a combination of conserved antiviral pathways, including RNAi, and novel uncharacterised mechanisms to target ONNV infections.

Materials and Methods

ONNV production and propagation

5’ONNVic-eGFP plasmid was kindly provided by Dr Brian Foy, Colorado State University. 5’ONNVic-eGFP infectious clones were generated as described in [19] with some modifications. RNA generated in vitro from the infectious clone template was purified using the RNeasy mini kit (Qiagen). RNA concentration and purity was ascertained using a Nanodrop (Labtech International). 2 µg of RNA were transfected into a confluent culture of VERO cells in a T75 flask using the Transmessenger transfection reagent (Qiagen). Cells were observed for cytopathic effects and GFP expression at 24 hours post transfection. At 72 hours post infection cells were scraped and filtered through a 0.22 µm filter, aliquoted and stored at −80°C. 250 µl of first passage 5’ONNVic-eGFP was used to infect a large culture of confluent VERO cells. At 72 hours post infection cells were scraped, filtered through a 0.22 µm filter, aliquoted and stored at −80°C. Second passage virus was used in all experiments.

Maintenance of G3 A. gambiae mosquitoes

Adult mosquitoes were maintained as described in detail by Sinden and co-workers [20]. In brief, mosquitoes were reared and maintained at 28°C, 65–70% relative humidity with a 12 hour light/dark cycle. Adult mosquitoes were fed on sterile filtered and autoclaved 10% fructose solution and used for experimental purposes when 1 or 2 days old.

Infection of adult G3 mosquitoes

Newly emerged female mosquitoes were inoculated with the required dilution of second passage 5’ONNVic-eGFP in MEM (Invitrogen), using a pulled capillary glass needle and a Nanoject (Drummond Scientific). Inoculated mosquitoes were kept in cohorts of 30–50 and maintained as described by [20]. Inoculated mosquitoes were double-contained to prevent escape.

QRT-PCR to assay viral titre in adult mosquitoes

Pools of 30 whole mosquitoes were homogenised in 200 µl of Drosophila Schneider’s medium (Gibco). Homogenates were centrifuged at 3000 g for 30 minutes at 4°C to pellet debris. Supernatant was transferred to a new 1.5 ml eppendorf tube and centrifuged at 5000 rpm for a further 30 minutes at 4°C. The supernatant was filtered through a 0.2 µm filter, and 140 µl of the filtrate was used for viral RNA extraction using the Qiagen viral
RNA extraction kit according to the manufacturer’s instructions. 10 μl of vRNA was used to generate cDNA using the Superscript II kit (Invitrogen). To ascertain the abundance of viral RNA, or viral genome copy number, an absolute quantification method was used. cDNA was generated from vRNA extracted from a sample with a known viral titre, calculated using standard plaque assay. A standard curve the sample was generated using neat, 1:5, 1:10, 1:50, 1:100 and 1:500 dilutions of cDNA. Qrt-PCR was carried out using SybrGreen reagents (Applied Biosystems) and primers against the nsP3 ONNV gene (Table S3). The standard curve was used to calculate the viral genome copy number of an unknown sample by mapping the CT value to that of the standard curve, giving the viral genome copy number.

Qrt-PCR to assay gene KD efficiency
Total RNA was extracted from pools of ~10 mosquitoes in TRIzol (Invitrogen) 4 days after dsRNA treatment. cDNA was generated from total RNA using the Superscript II kit (Invitrogen). Primers were designed against GOIs such that there was no overlap with dsRNA probes (Table S3) including the S7 gene that is constitutively expressed in the mosquito. Qrt-PCR was carried out using SybrGreen reagents (Applied Biosystems). cDNA input was normalised using the abundance of S7 in each sample. Once normalised, gene KD efficiency was calculated as a relative % decrease in transcript abundance compared to a control KD sample.

Plaque assay
Standard plaque assays were carried out as described in [21]. In brief, individual mosquitoes were homogenised in 270 μl of Drosophila Schneider’s medium (Gibco) and filtered through 0.22 μm filters. 10-fold serial dilutions of each sample were added in duplicate to confluent monolayers of VERO cells in 24 well plates and immobilised using an agar nutrient solution. Cells were stained after 4 days incubation at 37°C using 200 μl of 5 mg/ml Thiozolyl Blue Tetrazolium Bromide (MTT) (Sigma) in PBS. Plaques of dead cells were counted and used to calculate the plaque forming units (PFU)/mosquito.

Microarray hybridisation and analysis
Total RNA extracted from whole homogenates of A. gambiae mosquitoes was amplified and labelled using the Low RNA Input Amplification kit (Agilent, UK). In brief: 2 μg of total RNA was used in a random primed reverse transcription reaction to generate cDNA. After amplification by conversion to cDNA, cDNA was transcribed to copy messenger RNA (cmRNA) incorporating either Cy-5UTP (for the reference sample) or Cy-3UTP (for the test sample) fluorescent nucleotide analogs. cmRNA quality and labelling efficiency was assessed by spectrophotometry using a Nanodrop (Labtech International). If cmRNA yield was sufficient and Cy-3UTP or Cy-5UTP labelling was successful, 825 ng of RNA was hybridised to the Agilent 4X44K array in 2 x GEx-hybridisation buffer HI-RPM at 60°C for 17 hours. Hybridised slides were washed with GE wash buffer 1 at RT for one minute and GE wash buffer 2 at 37°C for one minute, to remove excess labelled cmRNA prior to scanning.

Microarrays were scanned using a GenePix semiconfocal microarray scanner (AXON Instruments, Foster City, CA). GenePix Pro 6.1 was used to record feature signal intensity, to eliminate local backgrounds, for grid alignment and manual inspection of feature quality. Average feature diameter was calculated and features lying outside three standard deviations of the mean were excluded from analysis. The ratio of feature intensity verses local and global backgrounds were calculated and features not exceeding background intensities were excluded from analysis. Features were normalised using Genespring 6.1 (Axon instruments) by locally weighted linear regression methods (Lowess). Feature intensities of the three biological replicates were averaged. T-test p-values were calculated, and normalised data was filtered to exclude data with p-values greater than 0.05. Data was further filtered to include only genes showing 2-fold and greater regulation. Candidate genes were selected based on several criteria, including gene ontology, and known roles of orthologous genes. Microarray data has been submitted to the open access Vectorbase database (www.vectorbase.org).

Design and production of dsRNA probes
Primers were designed (Table S3) for 200-600 bp sections of genes of interest, with a T7 promoter sequence (GAATTAA-TACGACTCAGTATAGGAGA) added to their 5’ ends. Polymerase chain reaction (PCR) was carried out using cDNA derived from A. gambiae mosquitoes and PCR products were sequenced to confirm correct amplification for each probe. PCR amplicons were used to synthesise dsRNA using the T7 MEGAScript kit (Ambion). Concentration of dsRNA was adjusted to 3 μg/μl and stored at ~80°C until use.

Co-infections with P. berghei
P. berghei ANKA clone 259c12 was maintained in Theiler’s original mice (Harlan, UK) as described in [20]. All animal work was carried out by Dr Tibehu Habtevetold and Kasia Sala. Mice were infected by intraperitoneal (IP) injection of 100–200 μl of P. berghei infected blood. For mosquito infections, three days after passage with infected blood mice were terminally anaesthetised with an intramuscular (IM) injection of 0.05 ml/10 g body weight of Rompun (2% stock solution, Bayer), Ketaset (100 mg/ml ketamine, Fort Dodge Animal Health Ltd) and PBS in a 1:2:3 ratio.

Newly emerged adult G3 mosquitoes were intrathoracically inoculated with ~1640 PFU 5′ONNVic-eGFP. Inoculated mosquitoes were maintained at 27°C for 48 h. Mosquitoes were starved of sugar for 4–5 hours prior to blood feeding. Mosquitoes were fed on a terminally anaesthetised P. berghei infected mouse, maintained at 19°C for 72 h post blood feeding to allow successful parasite development and were subsequently maintained at 27°C to allow for optimal viral replication. Unfed mosquitoes were removed between 24 and 48 h post blood feeding, when the blood bolus is clearly visible through the abdomen of the mosquito. Seven days post blood feeding, mosquito midguts were dissected and fixed in 4% PFA. Fixed midguts were mounted in Vectorshield (Vectorlabs) on glass slides with sealed coverslips. Live oocysts expressing GFP were counted using fluorescence, and melanised ookinetes were counted using light microscopy.

Statistics
For plaque assay experiments and P. berghei oocysts/ookinete quantification, results were subject to the Man Whitney U-test for statistical significance. Significance was accepted where P<0.001***, P<0.01**, P<0.05*. For analysis of changes in P. berghei melanisation prevalence, results were subject to the Chi Squared test for statistical significance, where P<0.001***. Statistical significance in microarray experiments was calculated using the T-test comparing normalised (Lowess) expression data. Differential regulation was considered to be fold change in expression was greater than 2 and P<0.05 over three biological replicates.
Ethics statement and approval of experimental procedures

This study was carried out in strict accordance with the United Kingdom Animals (Scientific Procedures) Act 1986. The protocols for maintenance of mosquitoes by blood feeding and for infection of mosquitoes with *P. berghei* by blood feeding on parasite-infected mice were approved and carried out under the UK Home Office License PL170/6347 awarded in January 2008 and PL170/7185 awarded in November 2010. The procedures are of mild severity and the numbers of animals used are minimized by incorporation of the most economical protocols. Opportunities for reduction, refinement and replacement of animal experiments are constantly monitored and new protocols are implemented following approval by the Imperial College Ethical Review Committee. The experimental procedures for the ONNV work were approved by the HSE and the Imperial College GM Safety Committee.

Results

*A. gambiae* infections with ONNV

Using an infectious clone of ONNV encoding enhanced GFP (5’ONNVic-eGFP) under the control of a duplicated viral subgenomic promoter (provided by B. D. Foy, AIIDL, Colorado state University [19]), we characterized infection of ONNV in adult G3 *A. gambiae* mosquitoes. Adult mosquitoes were intratracheally inoculated with ~1640 PFU/mosquito. Viral RNA (vRNA) was extracted from 10 pooled mosquitoes every day over 9 days and quantitative real-time PCR (qrt-PCR) was used to calculate the viral genome copy number/mosquito (Figure 1A). Viral titre increased slowly until 5 days post infection (DPI), when infection rapidly increased, peaking at 7–8DPI, and then subsequently decreased to low levels at 9DPI. Plaque assays using individual mosquitoes at 7DPI showed that the prevalence of infection was ~90% (data not shown). GFP expression was also monitored at 1, 4 and 9 DPI by fluorescence microscopy of live, cold anesthetized mosquitoes (Figure 1B). GFP expression, most commonly visible through the eyes (Figure 1C) and occasionally through the thorax, was visible in only ~20% of mosquitoes at 4DPI and ~25% of mosquitoes at 9DPI. The discrepancy in infection prevalence between plaque assays and GFP observations is attributed to the mosquito cuticle that provides a barrier to GFP detection and together with strong autofluorescence leads to underestimation of the infection prevalence in whole mosquitoes. In dissected mosquitoes at 7DPI, patterns of infection and tissue tropism were in agreement with those previously published using the same strain of *A. gambiae* mosquitoes and the same infectious clone of ONNV [19]. Additionally, GFP expression was commonly seen in the midgut musculature of infected mosquitoes (Figure 1D) similar to what has been previously observed in other vector-alphavirus combinations [22,23].

Genome-wide transcriptional responses

In order to investigate the responses of *A. gambiae* to systemic viral infection, we utilised a genome-wide microarray platform to profile gene expression during a time-course of ONNV infection of the hemocoele. Three time points were selected for analysis: 1DPI (representing initial introduction of virus into the hemocoele), 4DPI (where virus has replicated, is being released from infected cells and is infecting new tissues) and 9DPI (where infection levels have significantly dropped). Transcriptional profiling of whole mosquito homogenates from infected versus mock-infected mosquitoes revealed a large number of viral responsive genes. Initial exposure to virus (1DPI) triggered the differential regulation of 66 genes (53 upregulated and 13 downregulated), increasing to 211 genes (119 upregulated and 92 downregulated) at 4DPI and dropping to 23 genes (20 upregulated and 3 downregulated) at 9DPI (Figure 2). A full list of regulated genes is presented in Table S1. Genes were grouped into functional categories based on gene ontology (GO) terms, orthologous gene function and literature reviews. These categories span a wide range of cellular and physiological processes including metabolism, RNA degradation, signalling, and cell division; however, the most striking category pertains to genes with putative immune functions, particularly at 1DPI (30% of regulated genes at 1DPI, 18% at 4DPI and 26% at 9DPI).

Overall, 45 genes with putative immune functions were differentially regulated following ONNV infection. Grouping these genes based on gene ontology and putative function (Table S2) revealed genes with roles in all aspects of immunity, including pathogen recognition, complement-like proteins, immune signaling pathway components, humoral cascade regulators and effectors. The majority of genes (39/45) were upregulated, consistent with the hypothesis that viral infection triggers immune signalling in *A. gambiae*.

A surprisingly small number of genes from immune signalling pathways known to respond to viral infection in other invertebrates were differentially regulated; comparison of viral responsive genes with those downstream of the Toll and IMD pathway in *A. gambiae* [24] and the JAK/STAT pathway in *A. gambiae* (unpublished data) demonstrated very little overlap in gene expression indicating that these pathways are not activated by ONNV infection. In fact at 4DPI genes involved in the RNAi, JAK/STAT and IMD pathways were downregulated suggesting inhibition of these signalling pathways. Downregulated genes encode the Tudor-SN (TSD), a component of the RNAi RISC complex, the janus kinase IKB of the JAK/STAT pathway, and IKB, a positive regulator of the IMD pathway. In contrast to decreased IKB transcripts, DPT and CEC3 (AMPs thought to be downstream of the IMD pathway) were upregulated at 1DPI and 4DPI.

Genes encoding putative recognition receptors were upregulated at 1DPI and 4DPI; two MD2-like receptors (ML11/9), three galectins (GALE6-8), one fibrinogen-like protein (FREP5) and GNBP1 all increased in transcript abundance. Additionally a large number of genes encoding proteins implicated in humoral immunity were upregulated, consisting of LRIMs and complement-like Thioester-containing proteins (TEPs). Different LRIMs and TEPs were regulated during the different phases of infection; LRIM1/4 and TEP5 at 1DPI; LRIM7 and TEP4/9/10/12 at 1DPI and 4DPI; LRIM10 and TEP14 at 4DPI and LRBD7 at 4DPI and 9DPI.

A number of clip-domain serine proteases and their inactive homologs (CLIPs) and C-type lectins (CTLs) were upregulated including two known inhibitors of melanisation (CTLMA2 and CLIP12). The roles of the other regulated CLIPs and CTLs are not known, although they probably function in the modulation of signalling that regulates humoral responses. Genes encoding additional putative immune effectors were upregulated, the majority of which at 4DPI, including two hydrogen peroxidases, a glutathione peroxidase and three lysozymes. Additionally apoptosis related genes also responded to viral infection. Downregulation of the inhibitor of apoptosis-1 (IAP1) and upregulation of Caspase-6 (CASP6) at 4DPI suggests that apoptosis may be triggered.

Gene silencing and ONNV infection phenotypic analysis

Transcriptional profiling highlighted immune genes that respond to infection, however, whether these genes have genuine antiviral functions could not be inferred from expression profiling alone. To
identify genes that have roles in *A. gambiae* antiviral immunity we developed an RNAi and qRT-PCR based assay to measure the effects of gene knockdown (KD) on viral titres. Mosquitoes were co-inoculated with dsRNA corresponding to a gene of interest and 3000 PFU of 5'ONNVic-eGFP. The viral RNA genome copy number per mosquito was calculated 7 DPI using qRT-PCR. 19 genes were selected from the viral responsive immune genes identified in our transcriptional analysis and from the classical immune signalling pathways. DsRNA corresponding to *AgAGO2* and the ONNV nsP3 gene were included as positive and negative controls respectively, while dsRNA corresponding to the *Escherichia coli LacZ* gene was used as a reference to calculate percentage changes in viral infection loads. As expected KD of *AgAGO2* resulted in increased 5'ONNVic-eGFP titres and nsP3 silencing resulted in decreased 5'ONNVic-eGFP titres and nsP3 silencing resulted in decreased 5'ONNVic-eGFP titres.

**Figure 1.** *A. gambiae* G3 mosquitoes intrathoracically inoculated with 5'ONNVic-eGFP. (A) 10 inoculated mosquitoes were collected daily and qRT-PCR was used to ascertain viral genome copy number/mosquito. (B) Percent of inoculated mosquitoes showing GFP expression at 1, 4 and 9 DPI. (C) Brightfield (BF) and fluorescent (GFP) images of GFP expression in the head tissues through the ommatidia (O) of inoculated mosquitoes at 9 DPI and (D) Brightfield (BF) and fluorescent (GFP) images of GFP expression in nerves and/or muscle bands in the anterior- (A) and mid-gut (M) of inoculated mosquitoes. Error bars represent standard deviation of 3 biological replicates.

doi:10.1371/journal.pntd.0001565.g001
eGFP titres, indicating that our screening method was accurate (Figure 3). Interestingly, silencing of genes from the JAK/STAT and the Toll pathways (HOP, STAT1-2, PIAS, REL1, CACT), which are known to be involved in antiviral immunity in Aedes aegypti and D. melanogaster, as well as of the IMD pathway (REL2) did not have an effect on 5’ONNVic-eGFP titres or yielded data that were highly variable and therefore inconclusive. However, 5 of the 10 tested viral responsive immune genes identified in our gene profiling experiments appeared to be viral antagonists and were selected for further investigation: the putative recognition receptors ML1 and GALE8, and the antimicrobial peptides LYSCA, LYS6 and CEC3.

In order to confirm that the 5 genes identified in our qrt-PCR screen have a statistically significant impact on ONNV infection we carried out plaque assays to measure the viral titres in
individual gene silenced mosquitoes. Silencing 4 of the 5 tested genes consistently affected viral titres (Figure 4). ML1 KD resulted in a 6.2 fold increase (P < 0.0001), LYSC4 KD resulted in a 6 fold increase (P < 0.0001), LYSC6 KD resulted in a 5.4 fold increase (P < 0.001) and GALE8 KD resulted in a 2 fold increase (P = 0.0163) in median viral titres at 7 DPI. The prevalence of infection was similar for all genes tested (LacZ 89%, ML1 KD 95%, GALE8 KD 95%, LYSC4 KD 89% and LYSC6 KD 93%). Qrt-PCR was used to confirm the reduction of ML1 (91%), GALE8 (80%) and LYSC4 (82%) transcripts in mosquitoes after dsRNA treatment and to confirm the transcriptional profile of these genes after systemic infection (Figure S1); LYSC6 was not assayed.

**A. gambiae** co-infection with ONNV and *P. berghei*

Several recent studies have shown that the *A. gambiae* humoral immune system exists in a delicate state of balance that can be diverted to lysis or melanisation against malaria parasites [25,26]. To investigate whether the differential regulation of several humoral immune factors observed during infection with ONNV, including CLIPs, CTLs, LRIMs and TEPs, can influence this balance we utilised *A. gambiae* co-infections with ONNV and the rodent malaria, *P. berghei*.

Newly emerged female mosquitoes were injected with 1640 PFU 5’ONNVic-eGFP or mock infected and blood-fed 48 h later on a mouse infected with *P. berghei*. This experimental design resulted in parasite ookinetes traversing the midgut wall and entering the hemolymph approximately 4 days post ONNV infection. Seven days post blood-feeding the mosquito midguts were dissected and parasite oocysts and melanised ookinetes were counted. Additionally, midguts were scored for ONNV infection of the midgut musculature. Figure 5A shows the oocyst and melanised ookinete distribution in virally infected and mock infected mosquitoes. The results revealed that priming with ONNV results in an approximately 40% reduction in the number of live oocysts in the virally infected mosquitoes showing viral midgut infections, although this decrease

---

**Figure 3. RNAi and qrt-PCR based screening of 19 genes for antiviral function in ** *A. gambiae* **mosquitoes.** Mosquitoes were injected with dsRNA and ~3000 PFU of 5’ONNVic-eGFP concurrently, 30 mosquitoes were collected at 7 DPI, and qrt-PCR was used to ascertain viral genome copy number/mosquito. Values were normalised relative to the LacZ (non-specific) control and are given as a percentage relative to the LacZ titre. nsP3, a viral gene was included in the screen. Genes are divided into functional categories based on which immune signalling pathway they belong to, or their putative function. Genes with an asterisk* were selected for further characterisation.

doi:10.1371/journal.pntd.0001565.g003

**Figure 4. Four antagonists of 5’ONNVic-eGFP in ** *A. gambiae** **mosquitoes.** Mosquitoes were injected with dsRNA and ~3000 PFU/mosquito P1/P2V 5’ONNVic-eGFP concurrently. Individual mosquitoes were collected and subject the plaque assay at 7 DPI. Data was Log10 transformed. Bars represent the median from two independent biological replicates. P values indicate significance from Man Whitney U Testing of each gene KD compared to the LacZ control (P<0.05*, P<0.01**, P<0.001***).

doi:10.1371/journal.pntd.0001565.g004
was not statistically significant using the Man Whitney test. However, a statistically significant decrease in the numbers of melanised ookinetes was observed ($P = 0.001$). The prevalence of melanised ookinetes also significantly decreased from 35% in mock infected to 18.5% in virally infected mosquitoes (Figure 5B) ($P < 0.001$ using the Chi squared test).

**Discussion**

Viruses as obligate intracellular pathogens represent a unique challenge to the immune system and require sophisticated mechanisms of recognition and targeting. Completing their lifecycle within host cells and use of host cell membranes limits the number of signatures that the immune system can recognise as non-self. In this study we have attempted to elucidate the components of the immune system employed by *A. gambiae* mosquitoes to target ONNV through transcriptional profiling of infected mosquitoes and gene silencing experiments.

Our observations of slow viral replication and restricted tissue tropism within the *A. gambiae* mosquito host are consistent with those observed for ONNV infections in the past [19]; it has been suggested that the robust RNAi response observed in *A. gambiae* mosquitoes [27] may contribute to the poor vectorial capacity of these mosquitoes in comparison with other typical vector-virus combinations [6]. Although not carried out in this study, dissemination rates for ONNV infection in *A. gambiae* have also been shown to be low compared to other vector-virus combinations [19]. This indicates that *A. gambiae* is a poor vector of ONNV, and may not be the natural vector of the disease outside of epidemics [28].

Our transcriptional profiling of ONNV infected mosquitoes has identified a large number of viral responsive genes. A significant proportion of these genes have no known function, indicating that *A. gambiae* may utilise non-classical immune mechanisms to target viral infection. Of the putative immune genes that were viral responsive, a perhaps surprisingly small number were associated with the Toll or JAK/STAT pathways. It has been shown in *D. melanogaster* and *Ae. aegypti* that both the Toll and JAK/STAT pathways have important roles in antiviral immunity [4,5,29]. In our study ONNV infection fails to induce expression of components of these two pathways; in fact *HOP* is downregulated at 4DPI. Additionally, there is very little overlap between viral responsive genes and those known to be downstream of the Toll pathway in *A. gambiae* [24] and genes identified through microarray analysis of *HOP* KD *A. gambiae* mosquitoes (unpublished data). Indeed through our gene silencing experiments we have observed that activating or inhibiting both pathways has little effect on ONNV titres. These data indicate that not only does systemic ONNV infection fail to trigger Toll and JAK/STAT signalling, but that genes downstream of these two pathways do not target ONNV infection.

A possible contributing factor to the surprising difference observed between *Ae. aegypti* and *A. gambiae* responses to viral infection may be the route of infection used in the experimental designs. The JAK/STAT and the Toll pathways have been shown to be important in regulating flavivirus DENV midgut infection after an infectious blood meal, which is the natural route of infection. In our study,
intrathoracic inoculation was used to infect mosquitoes with ONNV in order to overcome the restricted tissue tropism and very limited dissemination rates observed following oral infection with ONNV, thus achieving higher levels and prevalence of systemic infection. Although a role of the JAK/STAT and the Toll pathway in midgut defense against ONNV is possible, our results demonstrate that once the virus enters the mosquito homocoeal, immune responses other than the JAK/STAT and the Toll pathways are involved in regulating systemic antiviral immunity. Other studies investigating immune responses of insects to alphavirus infection have also suggested or demonstrated that the JAK/STAT and Toll signalling pathways do not target alphaviral infection. Sanders et al [11] conducted microarray analysis of SINV infected Ae. aegypti; despite suggesting a role for the Toll pathway during early infection, they found no differential regulation of JAK/STAT or Toll pathway components. Fragkoudis et al [30] found that SFV in all likelihood does not trigger classical immune signalling pathways in Ae. aegypti cells, and in fact infection inhibits activation of the JAK/STAT, Toll and IMD pathways, probably through reducing host cell gene expression. The difference observed between immune gene regulation and function using Ae. aegypti/DENV and A. gambiae/ONNV may also be a feature of flavivirus versus alphavirus infection respectively.

In addition to Toll signalling, a second NF-κB signalling pathway, the IMD pathway, regulates numerous genes that target Plasmodium parasites and bacteria [24,31]. Our expression profiling shows that Ikß, a positive regulator of the IMD pathway, is downregulated at 4DPI, suggesting that the pathway may be inhibited. In contrast, upregulation of LRM1 and CEC3, which are downstream targets of the A. gambiae IMD pathway [31], as well as of a homolog of the AMP Diptericin that is downstream of the IMD pathway in Drosophila [32], may suggest activation of the pathway. Indeed a previous study has demonstrated that the IMD, but not the Toll pathway, has an antiviral function during SIN infection of Drosophila [33]. It is possible that the IMD, and not the Toll pathway, responds to alphavirus infection, with the reverse being true for flavivirus infection. Nevertheless, silencing REL2, the NF-κB factor of the IMD pathway, has no significant effect on ONNV titres, suggesting that the contribution of this pathway to ONNV infection, whether inhibited or activated, is minor.

RNAi has been demonstrated in a number of invertebrates to target and limit viral infection [6–10]. Our transcriptional profiling does not show any induction of expression of RNAi components, similar to observations of D. melanogaster infected with DCV [29] and Ae. aegypti infected with DENV [4]. Presumably the components of the RNAi pathway are constitutively expressed to levels sufficient to target replicating viruses. We confirm that RNAi is a key antiviral mechanism in A. gambiae mosquitoes through silencing of the nsP3 viral gene and inhibition of RNAi by silencing AgAGO2. Interestingly transcriptional profiling revealed that TNV, a component of the RISC complex in the classical RNAi pathway, is downregulated at 4DPI. It is possible that this downregulation is mediated by ONNV, as inhibition of RNAi would be advantageous for infection. ONNV and other alphaviruses are thought not to encode a direct suppressor of RNAi [34] as seen in other insect viruses such as the B2 protein of the FHV (Flock House Virus) [35]; however, it is possible that viral gene products may interfere with host gene expression. For example, 90% of Semliki Forest Virus (SFV; a closely related alphavirus) nsP2 protein localises to the nucleus of infected cells where its function is unknown but could modulate expression of host genes [36].

In addition to the downregulation of TNV, two further genes involved in the miRNA and piRNA pathways, DCR1 and AgAGO5, and a group of DEAD-box helicases are downregulated at 4DPI. There is evidence that the piRNA pathway has antiviral functions in Drosophila, as fuci mutant flies are more susceptible to WNV than wild-type flies [37]. The DEAD-box helicases, although having diverse functions in RNA metabolism, are closely related to RIG-I and the RIG-I-like receptors (RLRs) that have well defined roles in viral RNA recognition in mammalian systems [30]. In addition, the human DEAD-box helicase DDX5 also has antiviral roles, and multiple viruses have been shown to interact with this protein and modulate its function [39]. The downregulation of these genes suggests an intriguing function in antiviral immunity. However, silencing of three helicases, including DCR1, has no effect on ONNV titre.

In addition to the immune signalling pathways, A. gambiae mosquitoes have a humoral branch of the immune system that recognises and eliminates invading pathogens. A tertiary complex of two proteins of the LRIM family, LRM1 and APLIC, and the complement-like TEP1 has been shown to target invading Phasmodium parasites for lysis or melanisation [25,40,41]. As our transcriptional analysis showed that LRM1 as well as several other members of the LRIM and TEP families are upregulated following infection, we investigated whether these humoral responses triggered by viral infection can interfere with Phasmodium infections. ONNV and P. berghei co-infections of A. gambiae were timed such that parasites traverse the midgut and enter the hemolymph 4 days after ONNV infection, thus encountering virus-induced humoral immune responses in the hemolymph. Our results reveal that parasite melanisation is significantly inhibited in the presence of ONNV. These results are consistent with our transcriptional analysis that shows upregulation of two important negative regulators of melanisation at 4DPI: CTLMA2 and CLIP42 [42,43]. An observed simultaneous decrease in the number of surviving parasites is not dramatic suggesting that upregulation of LRM1 alone is not sufficient to cause significant parasite lysis, and that parallel upregulation of APLIC and the complement effector protein TEP1 would be needed. However, among additional TEPs upregulated by ONNV infection are TEP4 and TEP9, which have been recently shown to also form complexes with LRM1 [44]. It remains to be investigated whether the alternative LRM1/TEP complexes promote antiviral responses such as virus lysis or clearance of infected cells.

Four novel ONNV antagonists have been identified through our RNAi screen; MLI, GALE8, LISC4 and LISC6. MLI is one of two MD-2-like receptors upregulated by ONNV infection and a known P. falciparum antagonist [45]. The MD2 protein forms part of an LPS sensing mechanism in mammals [46,47]. In addition to responses to LPS, MD2-TLR4 signalling triggers the expression of pro-inflammatory cytokines in response to ebola envelope protein [48]. TLR4 signalling has also been linked to several other viral infections including the vesicular stomatitis virus, respiratory syncytial virus, mouse mammary tumor virus [48] and Kaposi sarcoma herpesvirus [49]; however the role of MD2 in these interactions is not clear. A TLR binding partner for the MLI5 has yet to be identified in flies and mosquitoes. One hypothesis is that the MLI proteins may act as an extracellular surveillance system that recognise viral PAMPs and lead to signalling via a Toll receptor. At least 10 Toll receptors have been identified to date in A. gambiae, but their role in immune responses is yet unclear [50].

Beta-galactoside binding galectins are found in many organisms and display a complex repertoire: the multiple isoforms and their glycan recognition properties [51]. There are 10 putative galectins in A. gambiae [50], 3 of which are upregulated by ONNV infection, GALE6-8. All three galectins are part of a mosquito specific expansion of the Galectin family, including also GALE4 and GALE5. This expansion maybe due to the haematophagous lifestyle of mosquitoes, and subsequent exposure to a disparate
group of blood-borne pathogens compared to D. melanogaster, including viruses. Therefore, the upregulation of several galectins in response to ONNV infection suggests that a common molecular signature is shared between these species. Galectins are known to function at several levels of antiviral defence, from initial recognition and blocking of envelope and fusion glycoproteins to the activation and amplification of the innate and adaptive immune responses [51]. In mammals, Galectin 1 cross-links the N-glycans displayed in the envelope proteins of Nipah and Hendra viruses (paramyxoviruses that induce syndecytia in infected cells), directly blocking cell infection and cell-cell fusion [51]. In addition, Galectin expression is regulated by Herpesvirus 1, Newcastle disease [52], Epstein Barr Virus [53], Hepatitis C virus [54] and Human papilloma virus (HPV) [55] while Galectin 3 secretion and carbohydrate binding increase upon Herpesvirus 1 infection [56].

Three lysozymes are also induced by ONNV infection and two of those, LYS4 and LYS6, appear to have antagonistic effects against ONNV infections. Although lysozymes are classical antibacterial proteins that function through perturbation of cell membranes [57], many of them are also shown to have antiviral immune functions including human urinary Lysozyme C, a lysozyme from chicken egg whites, human milk lysozyme and human neutrophil lysozyme, all of which have anti-Human Immunodeficiency Virus (HIV) activity [58]. In addition, a lysozyme from a marine organism is shown to inhibit Pseudo Rabies Virus (PRV) growth in cell culture [59]. Although, the mechanism of lysozyme antiviral activity is not clear, it is likely that they also function through membrane perturbation.

In summary, this study is the first step in elucidating the antiviral mechanisms of A. gambiae mosquitoes, and has revealed interesting differences between A. gambiae and other invertebrates. The finding that two pathways with known antiviral roles in other invertebrate-virus systems do not significantly modulate systemic ONNV infection indicates that A. gambiae may use other immune mechanisms to recognise and fight viral infections. Our data suggest that these mechanisms involve the complement-like branch of the humoral immune response, and that the melanisation response that is prominent in anti-parasitic immunity is suppressed. The antiviral immune response in A. gambiae is thus composed of some key conserved mechanisms to target viral infection such as RNAi but includes other diverse and possibly species-specific mechanisms.

Supporting Information

Figure S1 Qrt-PCR confirmation of virally responsive genes. The expression of 3 virally responsive genes ascertained by microarray analysis (white diamonds) was confirmed using qrt-PCR (black diamonds). cDNA was generated from RNA extracted from ONNVic-eGFP infected and mock infected (LacZ control) mosquitoes. Transcript levels are expressed as the fold change of those observed in the LacZ control. Error bars represent standard deviation of 3 biological replicates.

(TIF)

Table S1 Genes differentially regulated by 5'ONNVic-eGFP infection. Two-fold or greater fold change ratios are shown in black text for 1DPI, 4DPI and 9DPI. Fold change ratios less than 2-fold regulated that have passed all filters outlines in the materials and methods excluding filtering on fold change ratio, are shown in grey text. Putative functions/functional domains were derived from Gene ontology terms, Interpro domains and functions of orthologous genes (www.vectorbase.org).

(DOC)

Table S2 Immune genes regulated by viral infection at days 1, 4 and 9 post infection. Two-fold or greater fold change ratios are shown in black text for 1DPI, 4DPI and 9DPI. Fold change ratios less than 2-fold regulated that have passed all filters outlines in the materials and methods excluding filtering on fold change ratio, are shown in grey text. Putative functions/functional domains were derived from Gene ontology terms, Interpro domains and functions of orthologous genes (www.vectorbase.org).

(DOCX)

Table S3 Primers used for A. qrt-PCR and B. RNAi probe generation. A. Primers were designed using Primer3 for 50–150 bp sections of genes of interest. B. Primers were designed for 200–600 bp sections of genes of interest, with a T7 promoter sequence (GAATTAATACGACTCACTATAGGGAGA) added to their 5’ ends, ensuring no overlap between sections used for designing qrt-PCR primers.

(DOC)

Acknowledgments

The authors would like to thank Dr Brian Foy for provision of the infectious clone of ONNV used in this study.

Author Contributions

Conceived and designed the experiments: JW KEO GKC. Performed the experiments: JW. Analyzed the data: JW GKC. Wrote the paper: JW KEO GKC.

References

1. Beaty BJ (2005) Control of arboviruses diseases: is the vector the weak link? Arch Virol Suppl 19: 73–80.
2. Christophides GK, Vachou D, Katifotos FC (2004) Comparative and functional genomics of the innate immune system in the malaria vector Anophelidae gambiæ. Immunol Rev 198: 127–146.
3. Agaïse H, Perrimon N (2004) The roles of JAK/STAT signaling in Drosophila immune responses. Immunological Reviews 198.
4. Xi Z, Ramirez JL, Dinopoulos G (2008) The Aedes aegypti toll pathway controls dengue virus infection. PLoS Pathog 4: e1000988.
5. Souza-Neto JA, Sim S, Dinopoulos G (2009) An evolutionary conserved function of the JAK-STAT pathway in anti-dengue defense. Proc Natl Acad Sci U S A 106: 17841–17846.
6. Keene K, Foy BD, Sanchez-Vargas I, Beaty BJ, Blair CD, et al. (2005) RNA interference as a natural antiviral response to O’nyong-nyong virus (Alphavirus; Togaviridae) infection of Anopheles gambiae. PNAS 101: 13740–13745.
7. Campbell CL, Keene KM, Brackney DE, Olson KE, Blair CD, et al. (2008) Aedes aegypti uses RNA interference in defense against Sindbis virus infection. BMC Microbiol 8: 47.
8. Sanchez-Vargas I, Scott JC, Poole-Smith BK, Franz AW, Barbosa-Solomieu V, et al. (2009) Dengue virus type 2 infections of Aedes aegypti are modulated by the mosquito’s RNA interference pathway. PLoS Pathog 5: e1000299.
9. Myles KM, Wiley MR, Morazzani EM, Adelman ZN (2008) Alphavirus-derived small RNAs modulate pathogenesis in disease vector mosquitoes. Proc Natl Acad Sci U S A 105: 19930–19943.
10. Cirimotich CM, Scott JC, Phillips AT, Geiss BJ, Olson KE (2009) Suppression of RNA interference increases alphavirus replication and virus-associated mortality in Aedes aegypti mosquitoes. BMC Microbiol 9: 49.
11. Sanders HR, Foy BD, Evans AM, Ross LS, Beaty BJ, et al. (2005) Sindbis virus induces transport processes and alters expression of innate immunity pathway genes in the midgut of the disease vector, Aedes aegypti. Insect Biochem Mol Biol 35: 1393–1397.
12. Ramireza JL, Dinopoulos G (2010) The Toll immune signaling pathway controls conserved anti-dengue defenses across diverse Ae. aegypti strains and against multiple dengue virus serotypes Developmental and Comparative Immunology 34: 625–629.
13. Shore H (1961) O’nyong-nyong Fever; an epidemic virus disease in East Africa: one clinical and epidemiological observations in the northern province of...
Singh G, Popli S, Hari Y, Malhotra P, Mukherjee S, et al. (2009) Suppression of RNA silencing by Echko house virus E2 protein is mediated through its interaction with the PAZ domain of Dicer. FASEB J 23: 1843–1857.

Peranen J, Rikkomaki M, Liijäläasto P, Kaaraiainen L (1990) Nuclear localization of Semliki Forest virus-specific nonstructural protein nsP2. J Virol 64: 1810–1816.

Chotkowskii HL, Ciota AT, Jia Y, Paiga-Basagoiti F, Kramer LD, et al. (2008) West Nile virus infection of Drosophila melanogaster induces a protective RNAi response. Virology 377: 197–206.

Ulvila J, Hulmark D, Ramet M (2009) RNA Silencing in the Antiviral Inimate Immune Defence – Role of DEAD-box RNA Helicases. Scandinavian journal of Immunology 71: 136–140.

Schofer M (2010) Human DEAD-box protein 3 has multiple functions in gene regulation and cell cycle control and is a prime target for viral manipulation. Biochem Pharmacol 79: 297–306.

Baxter RH, Steiner S, Chezanov G, Levashina EA, et al. (2010) A heterodisperse complex of the LRR proteins LRRM1 and APLIC regulates complement-like immunity in Anopheles gambiae. Proc Natl Acad Sci U S A 107: 16617–16622.

Baxter RH, Steiner S, Chezanov G, Levashina EA, et al. (2010) Two mosquito LRR proteins function as complement control factors in the TEP1-mediated killing of Plasmodium. Cell Host Microbe 5: 273–284.

Ota MA, Christophides GK, Katats FC (2009) Effects of mosquito genes on Plasmodium development. Trends Parasitol 25: 3–9.

Schmitz AK, Yasine H, Katats FC, Ota MA (2009) Two C-type lectins cooperate to defend Anopheles gambiae against Gram-negative bacteria. J. biol. chem 284: 17616–17624.

Povelones M, Upton LM, Sala KA, Christophides GK (2011) Structure-Function Analysis of the Anopheles gambiae LRRM1/APLIC Complex and its Interaction with Complement C3-Like Protein TEP1. PLoS Pathog 7: e1002023.

Dong Y, Aguilar R, Xi Z, Warr E, Mengin E, et al. (2006) Anopheles gambiae immune responses to human and rodent Plasmodium parasite species. PLoS Pathog 2: e52.

Bryant CE, Spring DR, Gangloff M, Gay NJ (2010) The molecular basis of the host response to lipopoly-saccharide. Nat Rev Microbiol 6: 8–14.

Vilain A, Ilev DR, Monks BO, Homan C, Golenbock DT (2005) MD-2. Immunobiology 211: 437–447.

Okumura A, Puaa PM, Yoshimura A, Hardy RN (2010) Interaction between Ebola virus glycoprotein and host toll-like receptor 4 leads to induction of pro-inflammatory cytokines and SOCS1. J Virol 84: 27–33.

Lagos D, Vart RJ, Gratric F, Westorp SJ, Emanu V, et al. (2008) Toll-like receptor 4 mediates innate immunity to Kaposi sarcoma herpesvirus. Cell Host Microbe 4: 470–483.

Waterhouse RM, Kriukhtina E, Meisner S, Xi Z, Alvarez KS, et al. (2007) Evolutionary dynamics of immune-related genes and pathways in disease-vector mosquitoes. Science 316: 1738–1743.

Vasta GR (2009) Roles of galectins in infection. Nat Rev Microbiol 7: 424–438.

Aliakvand, J, Dalgleish C, Leffler H, Karlsson A (2004) Newcastle disease virus mediated suppression of Epstein-Barr virus specific T-cell immunity in classic Hodgkin lymphoma. Blood 110: 1326–1329.

Mengshol JA, Golden-Mason L, Arikawa T, Smith M, Niki T, et al. (2010) A crucial role for Kupffer cell-derived galectin-9 in regulation of T cell immunity mediated killing of Plasmodium. Cell Host Microbe 5: 273–284.

Garcia-Pineres AJ, Hildesheim A, Dodd L, Kemp TJ, Yang J, et al. (2009) Gene Function Analysis of the Anopheles gambiae LRIM1/APL1C Complex and its Regulation and Cell Cycle Control and is a Prime Target for Virus. Microbial Biochem 284: 17616–17624.

Chen IDF, Yang Y, Li Z, Zhao H, Guo Y, et al. (2009) Modulation of the immune response against an HIV-1 virus-like particle in human dendritic cells by the HIV-1 p66/51 protein. J Virol 83: 10303–10312.