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Communication

Transcriptome of the *Aedes aegypti* Mosquito in Response to Human Complement Proteins.

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Abstract

Aedes aegypti is the primary mosquito vector of several human arboviruses including dengue virus (DENV). Vector control is the principal intervention to decrease the transmission of these viruses. The characterization of molecules involved in the mosquito physiological responses to blood-feeding may help to identify novel targets useful in the design of effective control strategies. In this study, we evaluated the *in vivo* effect of feeding adult female mosquitoes with human blood containing either heat-inactivated (IB), normal serum (NB), and RNA-seq based transcript expression was compared against sugar-fed (SF) mosquitoes. In the *in vitro* experiments, we treated Aag2 cells with a recombinant version of the complement proteins (hC3 or hC5a) and compared transcript expression to untreated control cells after 24h. The transcript expression analysis revealed that human complement proteins modulate approximately 2,300 transcripts involved in multiple biological functions, including the immune system. We also found 161 up-regulated and 168 down-regulated transcripts differentially expressed when hC3 and hC5a were compared against the control untreated cells. We conclude that active human complement induces significant changes in the transcriptome of *Ae. aegypti* mosquitoes, which can influence the infective capacity of pathogens ingested during blood meals.

Keywords: human complement; *Aedes aegypti*; blood-meal; hC3; hC5a

1. Introduction

A single mosquito, *Aedes aegypti*, out of more than 3,500 mosquito species worldwide, is the vector responsible for major arboviral epidemics of yellow fever (YFV), dengue (DENV), chikungunya (CHIKV), and Zika (ZIKV) [1, 2]. Mosquitoes acquire the

viruses from a vertebrate host following the ingestion of an infected blood meal [3]. Once inside the mosquito midgut, viruses must overcome several barriers before they can be transmitted back to the vertebrate host [4]. The first barrier is the midgut, where the pathogen would need to reach permissive cells before it is attacked by the mosquito digestive enzymes and active factors contained in the blood meal that can cause lysis of the virions [4, 5]. Surviving pathogens must then penetrate the midgut tissue, replicate and reach the salivary glands, which represents the second barrier, and the last door to exit the vector through saliva during their next feeding on a susceptible vertebrate host [6]. Therefore, understanding the mechanisms that mediate the colonization of the vector tissues by the virus is vital to determining vector competence and transmission dynamics, and finding novel mechanisms to prevent disease transmission.

During the blood-feeding, human factors in plasma remain active for several hours inside the mosquito midgut, influencing mosquito physiology [7]. Among those factors, activation of the complement system may damage the insect tissue and reduce the population of microorganisms present in it, representing an important limiting factor on pathogen survival during their first hours inside the arthropod [8-10]. The human complement is a system comprised of more than 30 soluble proteins and membrane-bound receptors, mainly found in their inactive forms [11, 12]. Upon activation, they generate products that opsonize (or coat) microorganisms and promote their phagocytosis by immune cells, among several other mechanisms to directly eliminate the microorganisms, such as causing lysis through the formation of the membrane attack complex or MAC [13].

In humans, the complement system can be divided into three main pathways: classical (activated by antibodies), alternative (constitutively activated by autocatalysis of C3 protein), and the lectin pathways (characterized by serine protease enzymatic activity). The human complement proteins C3 (hC3) and C5 (hC5) are among the most important factors in this system. For instance, hC3 is the central player as the three conventional complement pathways converge [14] and the most abundant complement protein in serum [15]. hC3 convertases cleave this protein into hC3a and hC3b, which are able to interact with cell surfaces directly with IgG molecules leading to phagocytosis of foreign particles [15, 16]. Furthermore, the hC5a (a product of hC5 cleavage) works as a chemoattractant for neutrophils and macrophages, which can also enhance phagocytosis, stimulating the release of granules and activating coagulation [17]. These two complement factors (hC3 and hC5) are highly active in the mosquito midgut up to 3h after feeding [10], suggesting that they may still be able to exert their antiviral activity during that period.

Previous studies suggested that decreasing DENV replication in the mosquito may be related to expression changes of *Ae. aegypti* macroglobulin complement-related proteins (AaMCR) and anti-microbial peptides (AMP) [18], and that hC5a may interact with midgut uncharacterized proteins increasing the AMP defensin A [8]. However, the exact mechanisms leading to the observed complement-dependent decrease in infection remain

unknown. The objective of this study was to evaluate the effect of human complement on the transcriptome of *Ae. aegypti* mosquitoes after blood-feeding (*in vivo*) or after direct exposure with the cell line Aag2 (*in vitro*). Our current study suggests that human complement, hC3 and hC5a, induces changes in the transcriptome of *Ae. aegypti*, both *in vivo* and *in vitro*, and regulate a significant number of transcripts, including AMP, that are related to immune responses. We conclude that human complement induces protein expression changes in *Ae. aegypti* that could be targeted as antigens to decrease the mosquito survival and inhibit virus transmission. In relationship with the vector, the human host is a constant when we compared to new or emerging viruses, which are more prone to mutation. Our rationale to focus on mosquito proteins that are activated against human complement is that these, unlike the mosquito proteins that are arbovirus species or serotype specific, could represent alternative candidates for control interventions such as transmission blocking vaccines (TBVs).

2. Results

2. 1. Sequencing and read mapping

To understand the molecular interactions between the human complement and *Ae. aegypti* (Rockefeller strain), RNA-sequencing was used to explore the changes in the mosquito transcriptome. More than 135 million (135,411,293), Illumina 50 bp single-end directional reads (strand-specific), were sequenced across all samples (Table S1). Each *in vitro* test was represented by three libraries with an average of 9.13 million reads per library. The *in vivo* tests were each represented by two libraries with an average of 8.87 million reads per library. The results obtained from the mapping of processed reads (131,210,742) against *Ae. aegypti* LVP_AGWG AaegL5.2 showed a higher percentage of uniquely mapped reads using STAR (between 6.71 to 7.72% more between 6.71 to 7.72% more that with Hisat2); therefore, these were the reads that were used for expression analysis.

2. 2. Transcript differential expression in NB and IB fed mosquitoes (in vivo expreiments)

RNA-sequencing was conducted to explore the changes in the *Ae. aegypti* abdominal region transcriptome in response to oral ingestion of human blood containing either heat-inactivated serum (IB) or normal serum (NB) at 3 hours post-feeding. RNA-seq analyses showed that 70.1% (19,866/28,353 AaegL5.2) of annotated protein-coding transcripts were expressed in the mosquitos. A total of 11.9% (3,369/28,353) of annotated protein-coding transcripts were identified with differential accumulation (FC >4, $p<0.05$) between NB and sugar-fed (SF) control females, with 1,452 up- and 1,917 down-regulated. Interestingly, when comparing IB vs. SF mosquitoes, the latest had a slightly lower number of transcripts (11.5% - 3,270/28,353) differentially expressed, with 1,496 up- and 1,774 down-regulated, represented in 44 up-regulated more transcripts in the IB fed group than in the NB. We also observed 143 more down-regulated transcripts in the NB when compared to the SF group. However, a significant number of them, 733 up- and 795 down-regulated transcripts, were common in NB and IB when compared to sugar-fed mosquitoes. Finally, a lower number of transcripts, 8.3% (2,350/28,353) were differentially expressed when comparing NB vs. IB, with 1,065 transcripts up- and 1,285 down-regulated.

Gene Set Enrichment Analysis (GSEA) of molecular functions (MF) based on Gene Ontology (GO) was conducted on the 8.3% differentially expressed transcripts in response to NB vs. IB (Figure 1). Differentially expressed transcripts were highly clustered in several molecular functions such as protein binding and kinase activity for up-regulation. Some of the most interesting transcripts found up-regulated in the NB fed group were associated with functions as ubiquitin ligase complex (AAEL010793-RB, AAEL010147-RJ, AAEL007694-RE, AAEL004875-RB, AAEL003466-RG, AAEL013530-RB, AAEL007187-RB); proteins involved in oxidative stress and heme-binding (AAEL000342-RF (peroxidasin), AAEL023523-RD, AAEL007563-RG (Dual Oxidase: Peroxidase and NADPH-Oxidase domains), AAEL003762-RC (Protoporphyrinogen IX oxidase), AAEL005108-RA (manganese-iron (Mn-Fe) superoxide dismutase), AAEL005478-RN (flavohemoprotein B5/b5r), AAEL012580-RA (3-hydroxyisobutyrate dehydrogenase), AAEL010580-RA (3-hydroxyisobutyrate dehydrogenase, putative), AAEL003762-RC (Protoporphyrinogen IX oxidase), AAEL005400-RD (2-hydroxyacid dehydrogenase), AAEL010158-RB (cytochrome P450), AAEL004457-RD (cytochrome c) and AAEL010393-RA (ferritin subunit, putative).

In contrast, down-regulated transcripts in the NB compared to the IB were mainly associated with functions related to carbohydrate metabolism (AAEL002781-RG (galactokinase), AAEL009387-RA (hexokinase)); transport and storage of lipids (AAEL006982-RG (lipase), AAEL006667-RF (phosphatidyltransferase), AAEL007996-RA (centaurin alpha), AAEL003402-RI (sphingomyelin phosphodiesterase)); cell signaling (AAEL008847-RF (wingless), AAEL004846-RD (Protein lin-7 homolog), AAEL008679-RC (Alpha-tubulin N-acetyltransferase)); regulation of nitrogenous compounds metabolism (AAEL009099-RD (uridine cytidine kinase), AAEL011698-RB (mRNA (guanine-7-) methyltransferase), AAEL013653-RB (tata-box binding protein), AAEL000185-RD (eukaryotic translation initiation factor), AAEL004132-RE (Mediator of RNA polymerase II transcription subunit 31), AAEL004385-RB (UGA suppressor tRNA-associated antigenic protein)); as well as ~73 transcripts involved in transcription regulation.

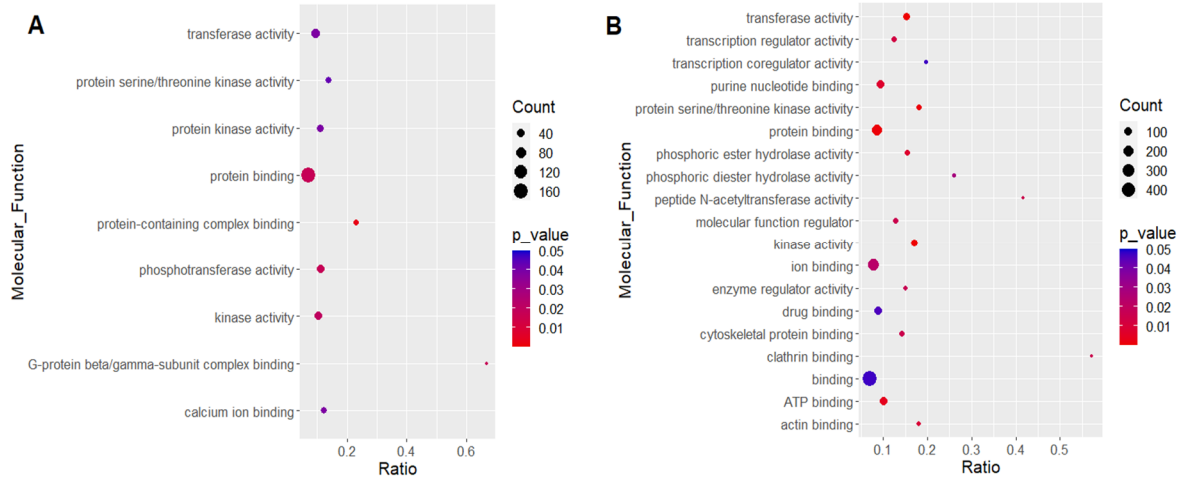

Figure 1. Gene Set Enrichment Analysis (GSEA) of molecular functions for differentially expressed transcripts after feeding with NB and compared to those fed with IB. **(A)** Up-regulated transcripts. **(B)** Down-regulated transcripts.

2. 3. Transcript differential expression in Aag2 cells upon treatment with hC3 and hC5a (in vitro experiments)

RNA-seq analyses showed that 54.4% (15,422/28,353 AaegL5.2) of all annotated *Ae. aegypti* protein-coding transcripts were expressed in hC3-treated, hC5a-treated, and control untreated Aag2 cells. A total of 2.6% (749/28,353) transcripts were identified with differential accumulation when comparing hC3-treated cells vs. control cells, with 324 and 425 transcripts up- and down-regulated, respectively. The up-regulated transcripts were enriched in phosphotransferase and oxidoreductase activities (AAEL008841-RC, AAEL008574-RB, AAEL003762-RD). While the down-regulated showed a significant increase in the kinase activity functions and binding proteins involved in the regulation of cellular processes (AAEL009041-RG, AAEL006510-RC, AAEL019921-RB, AAEL010926-RD) and the ubiquitin ligase complex (AAEL013530-RB, AAEL003466-RH, AAEL007694-RD, AAEL004875-RA, AAEL004697-RA).

The comparison between hC5a-treated cells and the untreated control cells revealed that 3.5% (981/28,353) of the transcripts had significant differential expression (504 up-regulated and 477 down-regulated). The up-regulated transcripts were enriched in ion binding, phosphotransferase activity, and nucleotide-binding functions; while the down-regulated transcripts were associated with functions such as actin-binding, cytoskeletal protein binding, GTPase regulator activity, and regulation of catalytic activity.

A total of 1.2% (329/28,353) differentially expressed transcripts, with 161 up- and 168 down-regulated, were in common between the hC3 and the hC5a treatments. The up-regulated transcripts presented enrichment in multiple molecular functions like binding, regulation, and oxidoreductase activity. GSEA of transcript differentially expressed in cells treated with either hC3 or hC5a in comparison to untreated cells is showed in Figure 2.

Figure 2. Gene Set Enrichment Analysis (GSEA) of molecular functions for the commonly up-regulated transcripts in Aag2 cells treated with hC3 and hC5a and compared against untreated control cells.

2. 4. Changes of the infection response transcripts (IRTs) to complement in the in vivo and in vitro experiments

Within the set of transcripts that were differentially expressed in *Ae. aegypti* after *in vivo* experiments, 142 transcripts (94 up and 48 downregulated) were involved in immune responses and other processes such as apoptosis and autophagy, these represent ~16% (142/886) of the total IRTs (Figure 3A, 3B and Table S2). From these, 77 transcripts were up-regulated after feeding with NB, 52 were found up-regulated after feeding with IB, and 35 were in common to both treatments. The top nine most abundant transcripts, with a fold change from 2.15 to 8.3 (Log2FC), encoded multiple protein families like serine-type endopeptidases Clip-Domain Serine Protease family C, family D, family E (AAEL000760, AAEL005718, AAEL007796, AAEL008668); Class B Scavenger Receptors (AAEL000234, AAEL000256, AAEL008370, AAEL009432); C-Type Lectin (AAEL011404, AAEL011407); Cecropins (AAEL029038, AAEL029041, AAEL029047); Defensins (AAEL003841, AAEL003857); IMD pathway signaling (AAEL012510, AAEL014734, AAEL015136); and one G-protein coupled receptor (GPCR) (AAEL002694).

Regarding the downregulated transcripts associated with IRTs, a total of 48 transcripts were founded (Table S2), of which 19 transcripts were common to both NB and IB fed mosquitoes. The down-regulated transcripts families involved principally fibrinogen related proteins (AAEL000726-RA, AAEL000749-RA); C-Type Lectin (AAEL000533-RA, AAEL000556-RA, AAEL018265-RB); serine-type endopeptidase inhibitor activity (AAEL004583-RG, AAEL019468-RD, AAEL026027-RB); trypsin (AAEL004543-RB, AAEL004996-RA, AAEL006425-RA, AAEL007818-RB, AAEL014579-RA, AAEL007818-RA, AAEL013713-RP); and Serine Protease Inhibitor (Serpin) (AAEL007420-RB, AAEL013936-RH, AAEL014141-RG). Interestingly, two transcripts down-regulated in IB but up-regulated in NB were AAEL009681-RB (Rhomboid-like protein) with a 5.7-fold increase and AAEL029038-RA (Cecropin) with a 10.2-fold increase.

In the *in vitro* experiments, the Aag2 cells treated with hC3 and hC5a also showed a change in the expression of IRTs (Table S2). The cells treated with hC3 compared to control cells presented a change in the expression of 13 transcripts (6 up- and 7 down-regulated) including the upregulation of caspase (AAEL014348-RF), Class B Scavenger Receptors (*i.e.* AAEL000234-RL, AAEL000256-RC), serine protease (AAEL002301-RB) and Sarm1 (AAEL014931-RD) transcripts. We observed a decrease of trypsins (AAEL007818-RA, AAEL013713-RO) and Serine Protease Inhibitors (serpins) (AAEL007765-RD, AAEL010769-RC). Also, when comparing Aag2 cells treated with hC5a compared to control cells, we observed 24 transcripts with differential expression, 12 up- and 12 down-regulated in hC5a-treated cells, specially we observed an increase in the expression of serine-type peptidases (AAEL013032-RC, AAEL023332-RC), serine-proteases (AAEL002301-RB, AAEL002601-RB), vesicular mannose-binding lectin (AAEL010584-RF), F-spondin (AAEL007889-RB), Toll-pathway signaling NF-kappaB (AAEL007696-RF), sarm1 (AAEL014931-RD) and Class B Scavenger Receptor (AAEL011222-RA, AAEL000234-RA, AAEL000234-RL). The downregulated transcripts were centered in trypsins (AAEL007818-RA, AAEL013713-RI), Serine Protease Inhibitor (Serpin) (AAEL014141-RD, AAEL010769-RC), serine-type endopeptidase inhibitor activity (AAEL019468-RE), Class B Scavenger Receptor (CD36 domain) (AAEL000234-RC, AAEL000234-RE, AAEL000234-RK, AAEL000234-RN) and the GPCR (AAEL002694-RB), amongst others.

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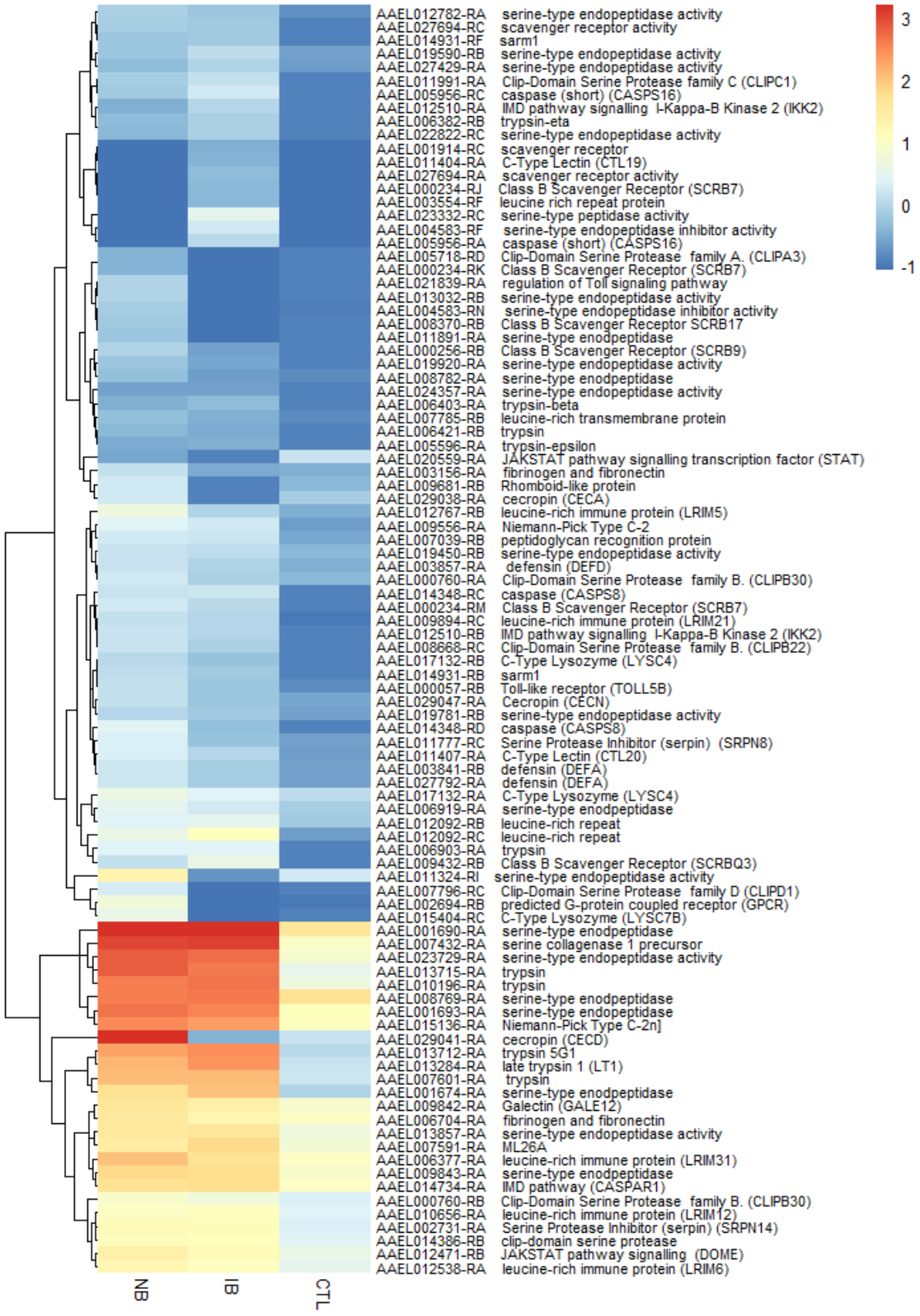

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232 **Figure 3A.** Heatmap showing the expression profile of 94 IRT transcripts found as up-regulated
233 after the *in vivo* treatments. Normal blood (NB). Heat-inactivated blood (IB). Sugar meal (CTL).

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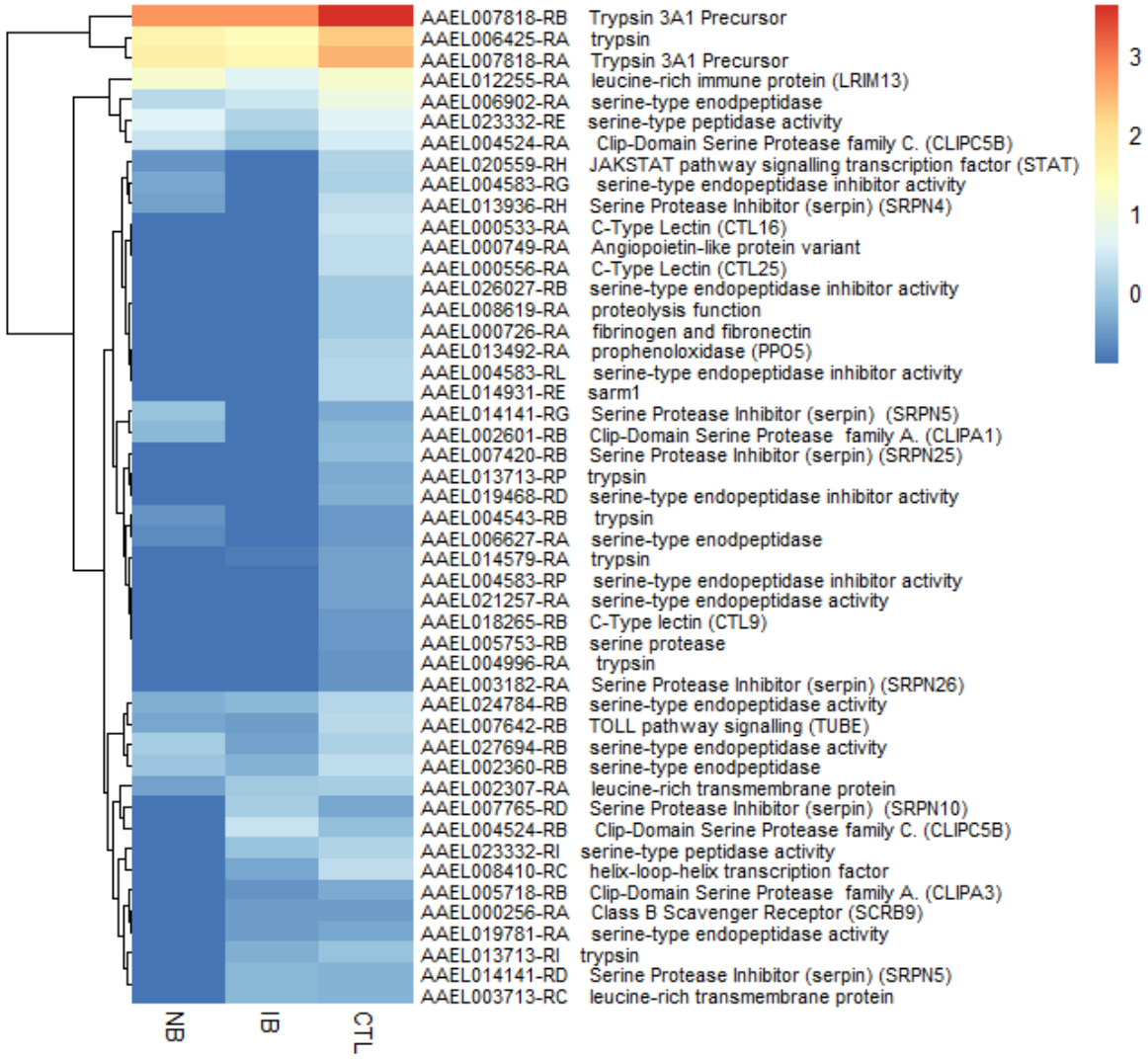

Figure 3b. Heatmap showing the expression profile of 48 IRT found as down-regulated in the *in vivo* treatments. Normal blood (NB). Heat-inactivated blood (IB). Sugar meal (CTL).

2.5. Shared transcripts between the in vivo and the in vitro assays

The comparison between transcripts found differentially expressed in live mosquitoes (*in vivo* assays) and those found in Aag2 cells (*in vitro* assays), shows 44 transcripts (21 up- and 23 down-regulated) in common for both experiments (Figure 4, Table 1).

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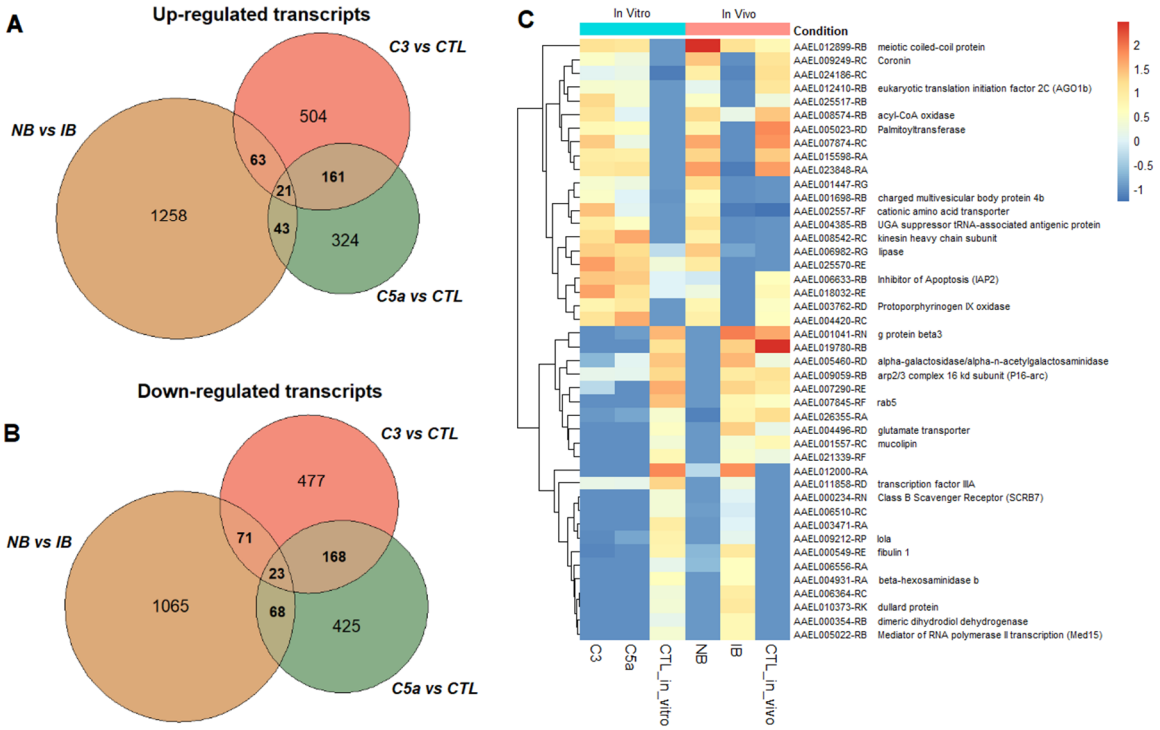

Figure 4. Differentially expressed protein-coding transcripts shared in response to both *in vivo* and *in vitro* experiments. Venn diagram of the up- (A) and down-regulated (B) transcripts. The heatmap (C) shows the clustering of the 44 (21 up- and 23-down) significantly regulated transcripts found in normal blood (NB), heat-inactivated blood (IB), and the *in vitro* experiments for treatments with hC3/hC5a and respective controls.

Table 1. The 44 shared differentially expressed protein-coding transcripts in response to treatment with human complement both *in vivo* and *in vitro*. The top of the table shows the up-regulated (positive FC values) and the bottom of the table shows the down-regulated (negative FC) transcripts. Gene Ontology Molecular Function and description (GO MF ID). Ctl. Denotes Aag2 untreated control cells.

Transcript_ID	**Gene Description** (Gene Ontology Molecular Function and description)	Log2FC		
		hC3/Ctl.	**hC5a/Ctl.**	**NB/ IB**
AAEL001698-RB	*Charged multivesicular body protein 4b* (GO:0007034, vacuolar transport)	2.80	2.06	5.46
AAEL007290-RE	*None* (None)	2.93	2.90	3.06
AAEL001447-RG	*None*	3.40	4.26	3.91
AAEL002557-RF	*Cationic amino acid transporter (GO:0022857, transmembrane transporter activity)*	3.41	2.91	5.27
AAEL005460-RD	*alpha-galactosidase/alpha-n-acetylgalactosaminidase (GO:0003824;GO:0016787;GO:0016798;GO:0004553, catalytic activity;hydrolase activity;hydrolase activity, acting on glycosyl bonds)*	3.78	3.75	3.54
AAEL025517-RB	*None (GO:0005524;GO:0004672, ATP binding; protein kinase activity*	3.79	3.47	5.92

AAEL012899-RB	*Meiotic coiled-coil protein, putative GO:0003690, double-stranded DNA binding)*	3.96	3.58	6.30
AAEL026355-RA	*None (GO:0005515, protein binding)*	4.25	3.23	4.85
AAEL008574-RB	*Acyl-CoA oxidase (GO:0071949;GO:0003997;GO:0050660;GO:0016627, FAD binding; acyl-CoA oxidase activity; flavin adenine dinucleotide binding)*	4.45	4.36	5.09
AAEL009249-RC	*Coronin (GO:0005515, protein binding)*	4.58	5.13	4.97
AAEL009212-RP	*Lola (GO:0005515, protein binding)*	4.86	4.88	6.08
AAEL007845-RF	*Rab5 (GO:0005525;GO:0003924, GTP binding; GTPase activity)*	4.88	5.16	6.89
AAEL001557-RC	*Mucolipin (GO:0005261, cation channel activity)*	4.89	2.70	5.76
AAEL005022-RB	*Mediator of RNA polymerase II transcription subunit 15 (Med15) (GO:0003712, transcription coregulator activity)*	5.01	5.26	8.12
AAEL003762-RD	*Protoporphyrinogen IX oxidase (GO:0016491;GO:0004729, oxidoreductase activity; oxygen-dependent protoporphyrinogen oxidase activity)*	5.15	6.23	3.13
AAEL015598-RA	*None (GO:0016787, hydrolase activity)*	5.21	2.97	2.24
AAEL019780-RB	*None (GO:0008410, CoA-transferase activity)*	5.27	5.19	2.62
AAEL005023-RD	*Palmitoyltransferase (None)*	5.33	6.30	5.14
AAEL021339-RF	*None (None)*	5.50	3.79	3.93
AAEL004931-RA	*Beta-hexosaminidase b (GO:0102148;GO:0004563;GO:0016787;GO:0016798;GO:0004553, N-acetyl-beta-D-galactosaminidase activity ;beta-N-acetylhexosaminidase activity)*	5.67	3.41	6.73
AAEL024186-RC	*None (GO:0003779;GO:0030276;GO:0005543, actin binding; clathrin binding; phospholipid binding)*	5.87	3.00	6.26
AAEL006633-RB	*Inhibitor of Apoptosis (IAP) (None)*	-7.22	-7.08	-4.47
AAEL006510-RC	*None (GO:0003723;GO:0008190, RNA binding; eukaryotic initiation factor 4E binding)*	-6.61	-6.04	-6.91
AAEL012000-RA	*None (None)*	-6.42	-6.29	-4.70
AAEL009059-RB	*Arp2/3 complex 16 kd subunit (P16-arc) (None)*	-5.86	-5.74	-5.88
AAEL001041-RN	*Guanine nucleotide-binding protein beta 3 (g protein beta3) (GO:0005515, protein binding)*	-5.72	-5.00	-4.14
AAEL000234-RN	*Class B Scavenger Receptor (CD36 domain) (None)*	-5.50	-3.75	-3.05
AAEL012410-RB	*Eukaryotic translation initiation factor 2C (GO:0003676;GO:0005515, nucleic acid binding; protein binding)*	-5.50	-4.20	-3.32
AAEL008542-RC	*Kinesin heavy chain subunit (GO:0005524;GO:0008017;GO:0003777, ATP binding; microtubule binding; microtubule motor activity)*	-5.04	-4.91	-4.09
AAEL023848-RA	*None (None)*	-4.87	-2.81	-6.23
AAEL007874-RC	*None (GO:0047372, acylglycerol lipase activity)*	-4.76	-4.63	-4.37
AAEL004496-RD	*Glutamate transporter (GO:0015293, symporter activity)*	-4.66	-4.52	-5.88
AAEL010373-RK	*Dullard protein (GO:0016791, phosphatase activity)*	-4.29	-4.16	-4.68

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AAEL006364-RC	*None (GO:0045127, N-acetylglucosamine kinase activity)*	-4.24	-4.11	-7.08
AAEL004420-RC	*None (None)*	-4.19	-4.06	-5.29
AAEL011858-RD	*Transcription factor IIIA (GO:0003676;GO:0008270, nucleic acid binding; zinc ion binding)*	-4.19	-4.07	-3.54
AAEL006982-RG	*Lipase (GO:0052689, carboxylic ester hydrolase activity)*	-4.18	-4.07	-2.50
AAEL025570-RE	*None (GO:0005524;GO:0003774;GO:0005515, ATP binding; motor activity; protein binding)*	-4.12	-6.57	-2.59
AAEL004385-RB	*UGA suppressor tRNA-associated antigenic protein (GO:0003824;GO:0016740;GO:0016785, catalytic activity; transferase activity; transferase activity)*	-4.11	-3.98	-5.09
AAEL018032-RE	*None (None)*	-3.97	-3.43	-6.07
AAEL000549-RE	*Fibulin 1 (GO:0005509;GO:0005515, calcium ion binding; protein binding)*	-3.75	-3.62	-4.69
AAEL000354-RB	*Dimeric dihydrodiol dehydrogenase (GO:0016491, oxidoreductase activity)*	-3.69	-3.56	-3.09
AAEL006556-RA	*None (None)*	-2.47	-2.43	-5.21
AAEL003471-RA	*None (GO:0003676;GO:0008270, nucleic acid binding; zinc ion binding)*	-2.44	-2.40	-3.73

3. Discussion

Our current study suggests that the ingestion of human blood containing active complement (NB group) by *Ae. aegypti* females leads to important changes in their transcriptome profile when compared to those fed with sugar (SF group). A significant number of these differentially expressed genes are linked to digestive processes, maturation of eggs, suppression of the response to stimuli, and even activation of the immune response [19]. Since complement activation may be detrimental to the midgut tissue, *Ae. aegypti* females have developed a mechanism to prevent such damage. One of them is the production of serine proteases that can specifically cleave and inactivate human complement proteins and catalases that reduce the oxidative stress caused by the hemoglobin heme group, extending the time of mosquito survival by days [20]. Serine-type endopeptidases such as trypsin are important for blood digestion and may be associated with DENV replication in mosquito midgut [5]. This study demonstrates that the activity of late-phase trypsin (5G1) was associated with a significant reduction of DENV2 replication in mosquito midgut [5]. Another study also showed that treatment of an infectious blood meal with a trypsin inhibitor reduced DENV-2 midgut titers, and delayed viral dissemination [21]. Our study revealed several serine-type endopeptidases up-regulated among the most abundant transcripts after feeding with human blood, while several transcripts for serine-type protease inhibitors were found downregulated, consistent we previous studies suggesting that these enzymes are important in blood meal digestion.

Our comparison between SF and NB fed mosquitoes showed a higher overall number of up-regulated transcripts when compared to those found in the comparison between the IB vs. SF groups fed mosquito suggesting that the presence of active complement induces a

different type of responses in the mosquitoes, especially when we see that more than 3,000 transcripts are differentially expressed in both groups but only roughly the half of those transcripts are shared between treatments. The comparison between NB and IB fed mosquitoes, showed that the significant number of up-regulated genes in the NB group are associated with iron metabolism and oxidative stress. Previous studies have shown that these functions are important in defense against pathogens and in protecting the arthropod against the blood-derived factors such as heme [22]. It is not surprising to find that transcripts related to iron metabolism were up-regulated in NB fed mosquitoes since this is the way blood is taken in nature, and mosquitoes will normally react to it as the beginning of the blood digestion. Regarding the oxidative stress in the midgut, previous studies suggest that the presence of heme significantly decreases reagent oxygen species in midgut as a protection mechanism against the oxidative stress induced by the heme overload product of hemoglobin digestion in the mosquito midgut [23]. Nevertheless, a recent study showed that the presence of heme in midgut induces significant changes in the expression of genes associated with energy metabolism and antioxidant activities, as it is also shown in our study [22].

Heat-inactivation of serum is mainly performed to denature complement proteins blocking the activation of all three complement pathways and avoiding the complement-mediated signaling in adjacent cells. Other studies have demonstrated that heat treatment of serum reduces phagocytosis and chemotactic signaling in human immune cells [24]. Early research on the effect of temperature on serum reported the formation of what was called the heat-labile and heat-stable anticomplementary activity or ACA. The studies revealed that while heat-labile ACA can be completely inactivated through serum heating at 56^0C for 30 min, the heat-stable ACA increases progressively with continued heating [25, 26], and it has been attributed to immunoglobulin aggregates [27]. In the current study, the transcripts highly represented as down-regulated in the NB group when compared to the transcripts in the IB inactivated fed mosquitoes were mainly associated with carbohydrate, lipid, and nitrogenous compounds metabolism, suggesting that heat inactivation of the serum may decrease signals in these midgut-associated pathways. It is thought that feeding the mosquitoes with heat-inactivated serum does not send the same “meal digestion” signals to the mosquito as when the blood is intact. Further studies are needed to investigate whether thermolabile ACA or other by-products of heat-inactivation induced the changes in transcripts observed in the present study.

One of the most noteworthy differences found in transcripts induced by active complement was the upregulation of AAEL009681-RB (Rhomboid-like protein) and AAEL029038-RA (Cecropin) with a fold increase higher than 5. Rhomboid proteins are associated with mitochondria homeostasis and cell signaling [28, 29]. In the case of the AMP cecropin, approximately ten genes have been reported in *Ae. aegypti* mosquitoes [30], and different cecropins can be induced by the microbiota or the presence of pathogens [31-33]. Our results suggest that human complement has an important role in the transcription of

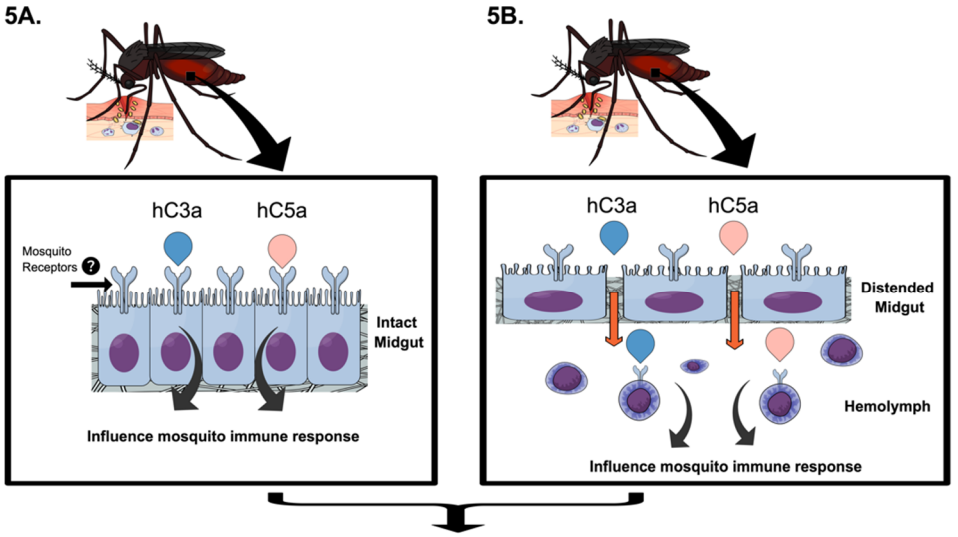
this cecropin. A previous study suggests that activation of complement-related proteins in *Ae. aegypti* mosquitoes induce the production of AMP, including cecropins [18]. Other studies confirm the importance of Toll-pathway induced cecropins in the control of DENV replication in the mosquito [34, 35]. Significant downregulation of cecropins upon infection with DENV [36] could represent a potential viral defense mechanism. Moreover, the increased expression of three defensins (AAEL003841-RB, AAEL027792-RA and AAEL003857-RA) and three cecropins (AAEL029038-RA, AAEL029041-RA, and AAEL029047-RA) in the NB group compared to IB, may indicate that human complement proteins directly influence the immune response of *Ae. aegypti* and corroborate our previous findings suggesting that activation of human complement impacts the synthesis of AMPs [8].

We also aimed to evaluate the direct effect of two specific human complement proteins, the hC3 and the hC5a on mosquito cells. First, the hC3 protein is one of the most abundant human complement factors in serum [37]. The hC3 can be physically adsorbed to tissue surfaces and the binding to membranes in its fluid form as C3(H2O) may trigger responses in human tissue [38]. Thus, our current study suggests that in the event of C3 interaction with the midgut cells, it may also induce significant transcriptome changes, as we observed more than 300 transcripts up-regulated and more than 400 transcripts down-regulated with an enrichment mainly in functions such as phosphotransferase and oxidoreductase activities.

The hC5a has shown an effect on DENV replication in our previous studies [8]. This molecule interacts with human cells through two possible receptors C5aR1, C5a receptor 1 (CD88), C5aR2, and C5a receptor 2 (C5L2) mainly found in bone tissue and immune cells [39, 40] where it can be associated with bone metabolism [41], induction of oxidative stress and modulation of cytokine production in immune cells [42]. In this study, we observed that transcripts modified by treatment with human hC5a are mainly associated with an increase of ion binding, phosphotransferase activity, and nucleotide-binding functions. We also observed a decrease of functions such as actin-binding, cytoskeletal protein binding, GTPase regulator activity, and regulation of catalytic activity. Our results also suggest that hC5a modified a larger number of transcripts than hC3, although only 329 transcripts were communally found modified in Aag2 cells after treatment with both complement proteins. Interestingly, we observed that the commonly up-regulated transcripts are associated with oxidoreductase activity and other enzymatic activities involved in mosquito metabolic processes such as ion binding and phosphotransferase activity, suggesting a direct effect of human complement proteins in mosquito physiology.

Recent studies showed that blood meal ingestion causes significant midgut distention allowing the exit of viral particles [43], and that the expression of molecules involved in the degradation/remodeling of the midgut extracellular matrix during the blood-feeding may impact the scape of viruses [44]. We speculate that the human complement proteins may

366 interact with mosquito cells in two ways, through receptors in the surface of midgut cells
367 before forming the peritrophic matrix (Figure 5.A) or by escaping through the distended
368 midgut cell layer during feeding (Figure 5.B) where hC3 and hC5 molecules in the
369 hemolymph could also interact with hemocytes.
370



371
372 **Figure 5:** Graphical summary of the effect of human complement proteins on mosquito cell
373 transcripts.

374 Complement activation during DENV infection in humans is a well-documented event,
375 and increased levels of complement proteins are found in DENV-infected patients
376 compared to uninfected individuals [45-47]. Interestingly, the vast majority of human
377 infections by DENV are asymptomatic (~70%) [48], and these individuals are associated with
378 lower systemic complement activation [49]. In contrast, higher activation of the complement
379 system and an excess of production of hC5a have been observed in severe dengue [50-52].
380 We hypothesized that complement activation in human blood may be detrimental to DENV
381 infection in the mosquito midgut and that viremic asymptomatic individuals may still be
382 infectious to mosquitoes and contribute significantly to virus transmission due to their
383 uninterrupted lifestyle [53] than severe DENV cases that are mostly confined to the hospital.
384 We speculate that lower complement activation in asymptomatic cases may be an important
385 factor in modulating infection and transmission in the mosquito vector.
386

387 The current study demonstrates the effect of human complement proteins on mosquito
388 physiology; however, we acknowledge that it has limitations. First, we only measured
389 transcripts at one point in time for both experiments, at 3h for the *in vivo* and at 24h for the
390 *in vitro* experiments. In addition, the *in vivo* experiment was only performed in duplicates.
391 We are planning to expand these experiments and measure additional time points in the *in*
392 *vivo* experiments (6, 12, 24, and 48h) in addition to compare transcripts in DENV infected
393 mosquitoes vs. those in non-infected to determine changes in the transcripts in relation to

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infection status. We believe that the findings of the current study support previous studies demonstrating the impact of human blood components in the physiology and immune response of arthropod vectors and highlights the importance of using normal non-inactivated human blood component to accurately evaluate vector competence. Furthermore, *Ae. aegypti* has a large number of transcripts involved in immune system processes; however, there is currently no curated list of them, to the best of our knowledge. Therefore, the proposed list established in this work of IRTs can serve as a frame of reference to establish a complete and refined list of immune-related genes of *Ae. aegypti* that can be used by the research community. In conclusion, our study shows that active complement induces significant changes in the transcriptome of *Ae. aegypti* mosquitoes with an important number of modulated genes involved in immune responses.

4. Materials and Methods

4.1. Human blood

The Institutional Review Board (IRB) approval for the collection of human blood from healthy volunteers was granted by the University of South Carolina IRB (IRB# Pro00045351). Human whole peripheral blood collected in EDTA was used in the experiments. Serum was separated by centrifugation, and the red blood cells (RBCs) were washed three times in 1 × DPBS and kept at 4°C until use. For the *in vivo* experiments, the inactivation of serum was accomplished by heating the serum for 30 min at 56°C. Inactivation of all complement pathways was verified using the Complement System Screen kit (Euro Diagnostica, Sweden) according to the manufacturer's instructions.

4.2. Maintenance of Aedes aegypti *adult mosquitoes and cell line*

Ae. aegypti mosquitoes (Rockefeller strain) were reared at 27±1°C, 75±5% RH, with a photoperiod of 16:8 h (L:D). Adults were maintained on 10% sucrose *ad libitum*. Female mosquitoes (n=60) 5 to 10 days-old were feed with a mixture of human RBC's in a 1:1 ratio with either heat-inactivated or normal non-inactivated human blood serum (Londono-Renteria et al., 2016). Aag2 *Ae. aegypti* cells, originating from embryonic cells [54], were grown at 28°C, Schneider *Drosophila* media supplemented with 10% heat-inactivated fetal bovine serum (Gemini, CA), 1% penicillin-streptomycin and 1% tryptose phosphate broth (Complete media) (Sigma, MO).

4.3. Mosquito blood-feeding

To measure the *in vivo* effect of human complement inactivation on mosquito physiology under experimental conditions, female adult mosquitoes were fed with either heat-inactivated blood serum (500uL inactivated plasma + 500uL of homologous packed RBC), or normal non-inactivated blood serum (500uL normal plasma + 500 uL of homologous packed RBC). Mosquitoes were fed for 30 min at room temperature using 1 mL of blood mixture in a Hemotek feeder maintained at 37°C. Engorged females were sorted in different cages and held under standard conditions. Age-matched sugar-fed mosquitoes were used as controls. Three hours (3h) post-feeding, whole abdomens were dissected and

transferred to 1.5-mL tubes in pools of 10 abdomens. The tissue was homogenized in RLT buffer (Qiagen, CA) with BME as the lysis buffer.

4.4. Aag2 cells exposure to human complement protein hC3 and hC5a

To measure the *in vitro* effect of the specific human complement proteins C5a and C3, we used the Aag2 *Ae. aegypti* cell line. Cells were seeded in a 24-well cell culture plate 24h before the experiment. Cells were treated with 1mg/mL of either recombinant hC3 (Abcam) or hC5a (R&D systems). Control cells contain complete media only. After 24h incubation at 28°C, cells were harvested using the lysis buffer described above.

4.5. Mosquito RNA isolation and sequencing

Mosquito RNA was extracted using a Quick-RNA miniprep kit (Zymo Research). Each *in vivo* experiment was conducted in duplicates for the feedings with sugar (SF), heat-inactivated (IB) blood, and normal blood (NB) (n=6). Each *in vitro* experiment was conducted in triplicates for Aag2 control untreated cells, cells exposed to hC3 and cells exposed to hC5a (n=9), for a total of 15 samples sequenced. A total of 3ng of RNA per sample was sent for sequencing to LC Sciences (Houston, TX), where the sample QC, library preparation (with enrichment for mRNA using poly(A) selection, single-molecule clonal amplification), and Illumina sequencing was performed.

4.6. RNA-seq differential expression analysis

Raw single reads were subjected to sequence quality control using FastQC v0.11.9 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and were quality filtered using Fastp v0.20.0 (Chen et al., 2018) to eliminate low quality and short reads (< 10 pb). High quality processed reads were mapped to the *Ae. aegypti* genome assembly AaegL5.2 [55] (https://www.vectorbase.org/ [55]) using STAR v2.7 [56] and Hisat2 v2.20 [57]. Mapped reads were counted using RSEM v1.3.3 [58].

The package NOIseq v2.31.0 [59] was used to perform differential expression analysis in the R software environment (https://www.r-project.org/), implementing the non-parametric approach NOIseqBio that improves the handling of biological variability specific to each gene, and is very successful in controlling the high false discovery rate (FDR) in experiments with biological replicates. The count filter was used to remove transcripts with Counts per Million (CPM) < 1 in the samples, which avoids noise from lowly expressed transcripts. The normalization method used was Trimmed Mean of M-values (TMM) approach. To identify those differentially expressed (DE) between comparisons, the q-value cut-off 0.95 was implemented, and only protein-coding transcripts with a fold change ≥ 4 (Log2FC ≥ 2) were analyzed.

The gene description and gene ontology (GO) terms of DE transcripts were obtained from VectorBase.org using the BiomaRt package v.2.44.1[60, 61]. The Gene Set Enrichment Analysis (GSEA) was performed using g: Profiler (https://biit.cs.ut.ee/gprofiler/gost). The transcripts related to infection response (IRTs), which include immunity and other processes such as small regulatory RNAs, apoptosis and autophagy, were identified considering the list of 477 transcripts obtained by *in silico* comparative genomic analyses and manual annotation that have established or putative associations with defense mechanisms published by Bonizzoni *et al*., [19]. Because this list of transcripts was generated with an

older genome assembly (AaegL1.2), only 243 of the 477 transcripts were found in the current assembly (AaegL5.2). With VectorBase.org, it was possible to convert some of the old IDs to the new ones, others have been lost. To look for new IRTs in the current assembly, the following functional annotations were used: GO:0002376 (immune system process), GO:0006955 (immune response), GO:0045087 (innate immune response), GO:0006959 (humoral immune response), GO:0006952 (defense response), GO:0006915 (apoptotic process), GO:0042981 (regulation of apoptotic process), GO:0008592 (regulation of Toll signaling pathway), GO:0008236 (serine-type peptidase activity), GO:0004252 (serine-type endopeptidase activity), GO:0004867 (serine-type endopeptidase inhibitor activity), GO:0008234 (cysteine-type peptidase activity) and GO:0004869 (cysteine-type endopeptidase inhibitor activity). The updated and curated list of IRTs contains 886 transcripts (Table S2).

The GSEA plots were generated using the ggplot2 package v3.3.0 [62, 63]. The heatmaps, clustered on expression profiles, were created to visualize changes in the experiment's profiles between transcripts. The z-score, shown as the scale key on the side of each figure, represents the normalized gene expression measurements, were 0 has no difference from the mean. To illustrate the transcripts expression, red color represents positive fold change (FC), indicating higher expression in the corresponding transcripts, and blue color represents negative FC, indicating decreased expression. The heatmaps were generated using the Pheatmap package v1.0.12 (https://cran.r-project.org/web/packages/pheatmap/index.html). The Venn diagrams represented the number of differentially expressed transcripts when comparing the different treatments (FC ≥ 4 and q-value > 0.95) and were generated using the Venneuler package v1.1-0 (http://www.rforge.net/venneuler/index.html).

Supplementary Materials: The following are available online at www.mdpi.com.

Table S1: RNA read summary in the *in vivo* and *in vitro* experiments.

Table S2: Differentially expressed immune-related transcripts (IRTs) found in the *in vivo* and *in vitro* experiments.

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