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Mosquito pericardial cells upregulate *Cecropin* expression after an immune challenge

Victor Cardoso-Jaime [a,b,1], Krystal Maya-Maldonado [a,b,2], Víctor Tsutsumi [b,**], Salvador Hernández-Martínez [a,*]

[a] Centro de Investigaciones Sobre Enfermedades Infecciosas, Instituto Nacional de Salud Pública. Av. Universidad 655, Santa María Ahuacatitlan, Cuernavaca, Morelos, C.P. 62100, Mexico

[b] Departamento de Infectómica y Patogénesis Molecular, Centro de Investigación y de Estudios Avanzados, IPN. Av. Instituto Politécnico Nacional 2508, Gustavo A. Madero, Ciudad de México, C.P. 07360, Mexico

A B S T R A C T

Most mosquito-transmitted pathogens grow or replicate in the midgut before invading the salivary glands. Pathogens are exposed to several immunological factors along the way. Recently, it was shown that hemocytes gather near the periostial region of the heart to efficiently phagocytose pathogens circulating in the hemolymph. Nerveless, not all pathogens can be phagocyted by hemocytes and eliminated by lysis. Interestingly, some studies have shown that pericardial cells (PCs) surrounding periostial regions, may produce humoral factors, such as lysozymes. Our current work provides evidence that *Anopheles albimanus* PCs are a major producer of *Cecropin 1* (*Cec1*). Furthermore, our findings reveal that after an immunological challenge, PCs upregulate *Cec1* expression. We conclude that PCs are positioned in a strategic location that could allow releasing humoral components, such as cecropin, to lyse pathogens on the heart or circulating in the hemolymph, implying that PCs could play a significant role in the systemic immune response.

1. Introduction

Vector-borne diseases are responsible for more than 700 000 deaths yearly, with mosquitos being of most significant concern since they play a role in disease transmission by carrying viruses and parasites (World Health Organization, 2017). Malaria remains the most important vector-borne disease, causing 400 000 deaths annually (World Health Organization, 2020). In Latin America, *Anopheles albimanus* is one of the most important *Plasmodium vivax* vectors (Fuller et al., 2012; Neafsey et al., 2015; Sinka et al., 2012). Despite of mosquitoes transmit pathogens to humans, they have a complex immune system, that protects them from bacteria, fungi, viruses and parasites (Clayton M. et al., 2014; King, 2020; Kumar et al., 2018; Moreno-García et al., 2014; Tikhe and Dimopoulos, 2021).

Plasmodium sporozoites migrate from the midgut to the salivary glands using the hemolymph flow (Hillyer et al., 2007). Interestingly, during this process, around 80–90% of sporozoites are eliminated; however, the mechanism by which this occurs is poorly understood (Hillyer et al., 2007). The fact is that sporozoites released into the hemolymph are exposed to different immune effectors. Phagocytosis, encapsulation, and nodulation (as part of the cellular immune response mediated by hemocytes), as well as humoral molecules such as antimicrobial peptides, lysozymes, and reactive oxygen species, all of them work together to neutralize pathogens (Clayton M. et al., 2014; King, 2020; Kumar et al., 2018; Miguel et al., 2014; Oliva et al., 2015). In *Anopheles gambiae* it has been reported that sporozoites are mostly accumulated around the periostial regions of the heart (King and Hillyer, 2012), where they are eliminated with the participation of hemocytes (sessile periostial hemocytes). A similar process occurs during bacterial infection (King and Hillyer, 2012; Sigle and Hillyer, 2016), suggesting that in addition to hemocytes, some heart components could be required to neutralize hemocoel-invading pathogens.

Pericardial cells are located at a strategic site of high hemolymph flow (flanking the ostia), where nutrients, wastes, signaling molecules, pathogens, hemocytes and immune response molecules enter the heart to be pumped-out and distributed throughout the mosquito body. In

* Corresponding author.
** Corresponding author.
E-mail addresses: vtsutsu@cinvestav.mx (V. Tsutsumi), shernandez@insp.mx (S. Hernández-Martínez).
[1] Current address: W. Harry Feinstone Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, USA.
[2] Current address: Department of Biology, Johns Hopkins University, Baltimore, Maryland, USA.

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addition, PCs are considered part of the mosquito renal system (analogous to kidney nephrons) because of their filtering functions (Crossley, 1972; Fife et al., 1987; Jones, 1954). Moreover, previous reports indicate that PCs express immune-related genes (Barillas-Mury et al., 1999; Danielli et al., 2000, 2003; Levashina et al., 2001; Lycett et al., 2004), suggesting that these cells may participate in the mosquito's immune response. In addition, we have reported that *An. albimanus* PCs have antimicrobial properties, implying that the heart may expresses immune-related genes such as lysozymes and cecropins (Hernández--Martínez et al., 2013b). We recently demonstrated that PCs express lysozyme c-1 in *An. albimanus* heart (Cardoso-Jaime et al., 2021), which supports the idea that additional immune-related genes, could be expressed by PCs. In *M. sexta* PCs was reported the expression of a cecropin (Dickinson et al., 1988), and in *D. melanogaster* PCs was evaluated the expression of *serpent*/dGATAb, an important transcription factor that regulates Cecropin A1 expression (Brodu et al., 1999). These previous evidences suggest that PCs could be involved in the insect response against pathogens, by producing humoral immune factors.

The current work provides additional evidence that *An. albimanus* PCs participate in the immune response, by expressing *Cecropin 1* (*Cec1*), supporting the idea that mosquito PCs are immunocompetent cells, which also can act as a connection between the circulatory and immune systems.

2. Materials and methods

2.1. Mosquitoes and microorganisms

The *Anopheles albimanus* Tapachula strain mosquitoes were obtained from the insectary of Instituto Nacional de Salud Pública (INSP), Cuernavaca, Morelos, México. Larvae stages were fed on pulverized kitty cat food pellets (Whiskas), and adult mosquitoes were fed on cotton pads soaked with 10% sucrose solution. Insects were maintained at 28 °C and 80% relative humidity (RH) with 12 h light/12 h dark photoperiod as previously described (Hernández-Martínez et al., 2019). For all experiments, female mosquitoes three days old were used.

Immune challenges were performed with *Saccharomyces cerevisiae*, *Micrococcus luteus*, and *Escherichia coli* (all from Sigma, St. Louis, MO). To prevent their proliferation all microorganism used were heat killed by boiling the samples during 10 min as previously described (Hernández-Martínez et al., 2013a).

2.2. Mosquito injections and heart dissection

Mosquito injections were performed as previously described (Hernández-Martínez et al., 2013b). Briefly, fine needles were made with 100 μl micro-capillary glass tubes using a pipette puller P-30 (Sutter Instrument, Novato, CA), and mounted on a syringe pump (Drummond, Broomall, PA). Groups of 20 mosquitoes per treatment were cold-anesthetized on ice, then were injected with 0.25 μL RPMI 1640 culture medium (Gibco, Grand Island, NY) containing 4×10^3 *S. cerevisiae* cells/μL, 34×10^3 *E. coli cells*/μL or 34×10^3 *M. luteus* cells/μL, all of them were heat killed. As control, mosquitoes were injected with RPMI alone. The injected mosquitoes were maintained 12 h at 28 °C with 80% RH, then hearts were obtained using an entomological needle as previously described (Cardoso-Jaime et al., 2021; Hernández-Martínez et al., 2013b). Newly obtained hearts were washed in 20 μL PBS [140 mM NaCl, 2.6 mM KCl, 1.5 mM KH_2PO_4, 20.4 mM Na_2HPO_4, pH 7.2 (all of them of Sigma, St. Louis, MO)], where most of fat body attached to the heart was removed. Then the hearts were transferred to a new drop of PBS to continue with two additional washes. Clean tissues were used for different assays. Additionally, the first drop of heart washes (from 20 mosquitoes) containing fat body was recovered in 800 μL of Trizol LS (Invitrogen, Carlsbad, CA). Three independent experiments (biological replicates) were performed in all treatments.

2.3. RNA isolation, cDNA synthesis and qRT-PCR

The RNA was obtained as previously described (Cardoso-Jaime et al., 2021). Twenty female mosquito hearts were collected in TRIzol Reagent (Invitrogen, Carlsbad, CA). RNA precipitation step was done in 250 μL of 3M sodium-acetate at pH 5.2, 5 μg of linear acrylamide (RNA carrier) and 1:4 absolute ethanol (Sigma, St. Louis, MO), 2 h at −20 °C. The precipitated was centrifuged at 12000 g, 30 min at 4 °C. The pellet was washed twice with 70% ethanol. The RNA pellet was resuspended in 10 μL of DEPC-water (Invitrogen, Carlsbad, CA).

cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Carlsbad, CA). One hundred nanograms of RNA treated with DNase I (Thermo Fisher Scientific, Carlsbad, CA) were used as a template on a final reaction volume of 20 μL. The qRT-PCR assays were performed using Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific, Carlsbad, CA). Each reaction was performed with 0.4 μL of cDNA reaction (per sample), 0.125 μM of primers forward (Fw) and reverse (Rv) each one. Sequences of primers were: Cecropin 1 (VectorBaseID: AALB006948), Fw_Cec1 (AGTGGACGCTGGTTTTCTCAA), Rv_Cec1 (ATTTGCCAAGTGCCTTCACG); GAPDH (VectorBaseID: AALB000739), Fw_GAPDH (ATGCCATCAAGGAGAAGGT), Rv_GAPDH (TTCACGAAGGTATCGCTCAG) (Primers were obtained from OligoT4, Irapuato, MX). Sample reactions were run in a ViiA7 Real Time PCR System (Applied Biosystems). Relative quantification of mRNA was performed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), with the results of three independent experiments and three replicates per sample each one. The GAPDH gene was used as reference gen and naïve group as the reference sample.

2.4. Hemocyte fluorescence labeling

Hemocytes were labeled as previously reported (Hernández-Martínez et al., 2017; King and Hillyer, 2012), but with some modifications. Briefly, mosquitoes (12h post-challenge) were injected with 0.25 μL of a solution of 75 mM CM-Dil (Vybrant CM-DiI Cell-Labeling Solution, Invitrogen, Carlsbad, CA) in RPMI. The mosquitoes were maintained at 28 °C and 80% RH for 20 min, then they were fixed by injecting 1 μL of 16% formaldehyde into the hemocoel. After 10 min, the abdomens were dissected and washed three times on a PBS drop, then were mounted with DAPI fluoromont G as anti-fading medium (EMS, Hatfield, PA). Samples were analyzed in an epifluorescence microscope Eclipse 80i (Nikon, Japan).

2.5. RNA FISH-STICs

Cec1 mRNA was detected *in situ* by RNA FISH-STICs, using a protocol previously described by (Sinnamon and Czaplinski, 2014). This methodology allows detecting mRNA *in situ* using three probes (one of them fluorescent), which interact with each other allowing the hybridization of a large amount of fluorescent probes in a short molecule of mRNA (Supplementary Fig. 1). The sequences of the secondary and tertiary probes were the same as those previously reported (Sinnamon and Czaplinski, 2014), the primary probes contain a modified sequence region that is complementary to *Cec1* mRNA sequence (Supplementary material 1) (Primary and secondary probes were obtained from IDT, Coralville, IA; and tertiary probe from Oligo T4, Irapuato, Mexico). We tested the specificity of probes by making different combinations of probes. Abdomens were used to test them because it contains the fat body which is the main producer of cecropins. We found that just tissues containing the three probes show signal, being fat body and PCs the tissues with high fluorescence as we expected (Supplementary Fig. 2).

The original protocol from Sinnamon and Czaplinski (2014), was adapted for mosquito tissues. Cold-anesthetized mosquitoes were placed on a PBS drop, and abdomens were split of thorax. The gut was removed, and the abdomen was cut along the left of lateral membrane and fixed in

500 μL of 4% formaldehyde for 30 min at RT. Samples were washed three times with PBS-T (PBS, 0.1% Tween-20), and permeabilized-dehydrated in ascending methanol concentrations (PBST/methanol; 7:3, 1:1, 3:7) for 5 min each, followed by absolute methanol for 10 min at RT. Immediately, the abdomens were rehydrated with descending methanol concentrations (PBS-T/methanol; 3:7, 1:1, 7:3) for 5 min each, and finally washed 4 times with PBS-T for 5 min each at RT.

The permeabilized-rehydrated abdomens were incubated in wash buffer [WB: 4XSSC, deionized 35% formamide (both from Invitrogen, Carlsbad, CA), 0.1% Tween-20 (Sigma, St. Louis, MO)], 10 min at 37 °C. WB was replaced by 100 μL of hybridization buffer [HB: 4XSSC, 35% deionized formamide, 0.1% Tween-20, 2 mM vanadyl ribonucleoside and 20 μg/mL BSA (both from New England BioLabs, Ipswich, MA), 1 mg/mL salmon sperm DNA and 100 mg/mL dextran sulfate sodium (both from Sigma, St. Luis, MO)] containing 1 μM of each primary probes. Samples were incubated for 16 h at 37 °C in dark. After the first hybridization, HB was removed, and abdomens washed twice with 500 μL of WB, 30 min each at 37 °C in dark, followed of incubation in 100 μL of HB containing 1 μM secondary probe, 3 h at 37 °C in dark. The abdomens were washed twice with 500 μL of WB, 30 min each at 37 °C in dark. For the third hybridization, 100 μL of HB containing 1 μM tertiary probe were added to abdomens followed by incubation for 3 h at 37 °C in dark. Finally, HB was removed, and the abdomens washed three times with 500 μL of WB, 30 min each at 37 °C, followed by five washes with PBS-T for 5 min each at 37 °C in dark. Abdomens were mounted on a glass slide using DAPI fluoromont G as anti-fading medium. Confocal C2 system attached to an E−600 microscope (Nikon, Japan) and NIS elements Nikon software were used to obtain single-projection images, and the image analysis was done using Fiji ImageJ software.

2.6. Statistical analysis

The qRT-PCR data were analyzed using GraphPad Prism version 5.0. To get statistical differences One Way ANOVA followed by Tukey's Test were used.

3. Results

3.1. An. albimanus heart express Cec1

Previously, we reported a transcriptomic analysis that suggests the *Cec1* expression on *An. albiamus* heart (Hernández-Martínez et al., 2013b; Martínez-Barnetche et al., 2012). As a first step toward validating this information, we evaluated the expression of *Cec1* by qRT-PCR on samples gotten from isolated hearts, whole body, and fat body, all of them from untreated mosquitoes. The results indicate that *Cec1* basal expression is higher in heart than in fat body or whole body (1.6-fold) (Fig. 1A). On the other hand, the newly obtained hearts may contain remnants of the fat body, which is the main producer of AMPs (Bulet and Stöcklin, 2005; Clayton M. et al., 2014; Dickinson et al., 1988; García Gil de Muños et al., 2008; Lowenberger et al., 1999), however, it was easily removed after being washed carefully (Fig. 1B–D), indicating that *Cec1* expression is derived from heart-associated cells. Together, these findings point to the heart as an important organ in mosquitoes that produces *Cec1*.

3.2. Cec1 expression is upregulated in An. albimanus heart after an immune challenge

One feature of an immunocompetent organ, is its ability to respond to microbes (Zuk and Stoehr, 2002) through humoral factor upregulation as antimicrobial peptides (Lemaitre et al., 1997). To demonstrate the heart's response against an immune challenge by upregulation of *Cec1*, we used qRT-PCR to examine the relative expression of *Cec1* in hearts from mosquitoes injected with *M. luteus*, *E. coli*, and *S. cerevisiae*. The results (Fig. 2) showed that *Cec1* expression was upregulated in presence of all used microorganisms tested, *E. coli* (9.7-fold), *M. luteus* (39.4-fold) and *S. cerevisiae* (45.8-fold), indicating that the heart could be an essential immunocompetent organ in mosquitoes.

Previously, it was reported that systemic infection on *An. gambiae* induces hemocytes accumulation around the heart (King and Hillyer, 2012), which also expresses cecropins (Bartholomay et al., 2004; Choi et al., 2012). Similarly, to *An. gambiae*, we observed hemocytes accumulated on the *An. albimanus* heart after the immune challenge with any

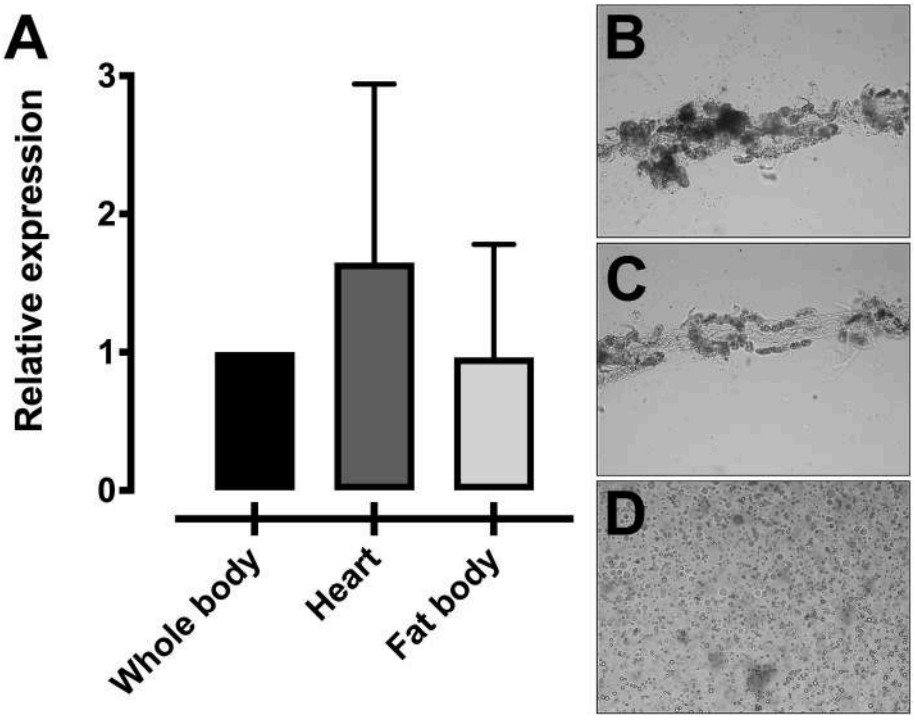

Fig. 1. *Cec1* is expressed in *An. albimanus* heart. The transcriptional *Cec1* expression in the whole body, heart, and fat body was measured by qRT-PCR (A). Newly obtained heart samples containing fat body tissue attached to them (B). Heart samples, after carefully washed (fat body-free) were used in qRT-PCR (C). Fat body cells recovered from heart washes (D). Each error bar presents the mean ± SD. Data were analyzed by One Way Anova followed by Tukey's test, no significant values were found. Values from three independent biological experiments. Each sample was obtained from 20 hearts or fat body tissues of 3 days-old non-treated females. Pericardial cells (PCs), fat body (FB), dorsal vessel (DV). Phase contrast microscopy, scale bar = 200 μm.

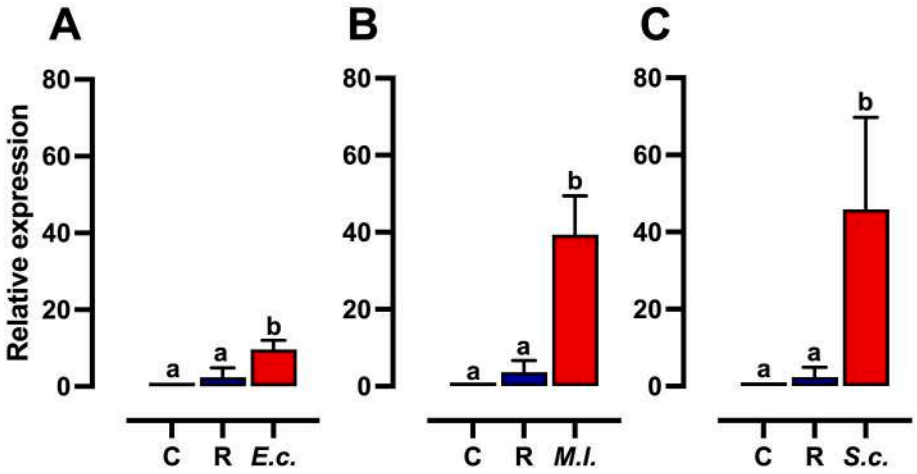

Fig. 2. *Cec1* expression is upregulated after an immune challenge. *Cec1* expression was measured in heart samples from female mosquitoes at 12 h post-injection with A) *E. coli* (*E.c*), B) *M. luteus* (*M.l.*), C) *S. cerevisiae* (*S.c.*). Heart samples from mosquitoes non-injected (C) or RPMI-injected (vehicle, R), were used as controls. The highest *Cec1* expression was observed in the heart from mosquitoes challenged with *S. cerevisiae*. *GAPDH* was used as a housekeeping gene, and relative expression was obtained using as reference the *Cec1* expression of heart samples from non-injected mosquitoes. Each bar represents the mean ± SD of three independent experiments. Each sample was obtained from 20 mosquito hearts per treatment for each experiment. Values significantly different by Tukey's test after One Way Anova are indicated by different letters ($P < 0.05$).

of the microorganisms used (Fig. 3). Even though hemocytes attached to the heart are weakly joined and are easily removed after washing, we found few hemocytes remained attached to the heart after several washes. However, no discernible variations in the number of the hemocytes attached to the heart were observed in any treatment after washing (Supplementary Fig. 3), but important differences in *Cec1* upregulation were observed. This shows that *Cec1* upregulation after a microorganism challenge is coming from the heart, rather than the accumulation of periostial sessile hemocytes.

3.3. *Cec1 is highly expressed on pericardial cells*

The last results demonstrated that *An. albimanus* heart expresses *Cec1*, although they do not define which heart cell type is expressing *Cec1*. To identify the heart cells expressing *Cec1*, we adapted the RNA FISH-STICs approach (Fluorescence In Situ Hybridization with Sequential Tethered and Intertwined ODN Complexes). According to the findings in Fig. 4, PCs are the only cell type from the heart constitutively expressing *Cec1*. In addition, *Cec1* expression was upregulated in heart samples from *S. cerevisiae*-challenged mosquitos, which agrees with the results obtained by qRT-PCR (Fig. 2). Interestingly, *Cec1* expression was higher in hearts than in fat body (Fig. 4) in both conditions, which reinforce the results in Fig. 1. In addition, PCs from *S. cerevisiae*-injected showed an evident increase in size (Fig. 4), however no quantitative evaluations were performed. Taken altogether, our findings show that PCs are among the most important *Cec1* producer during the mosquito

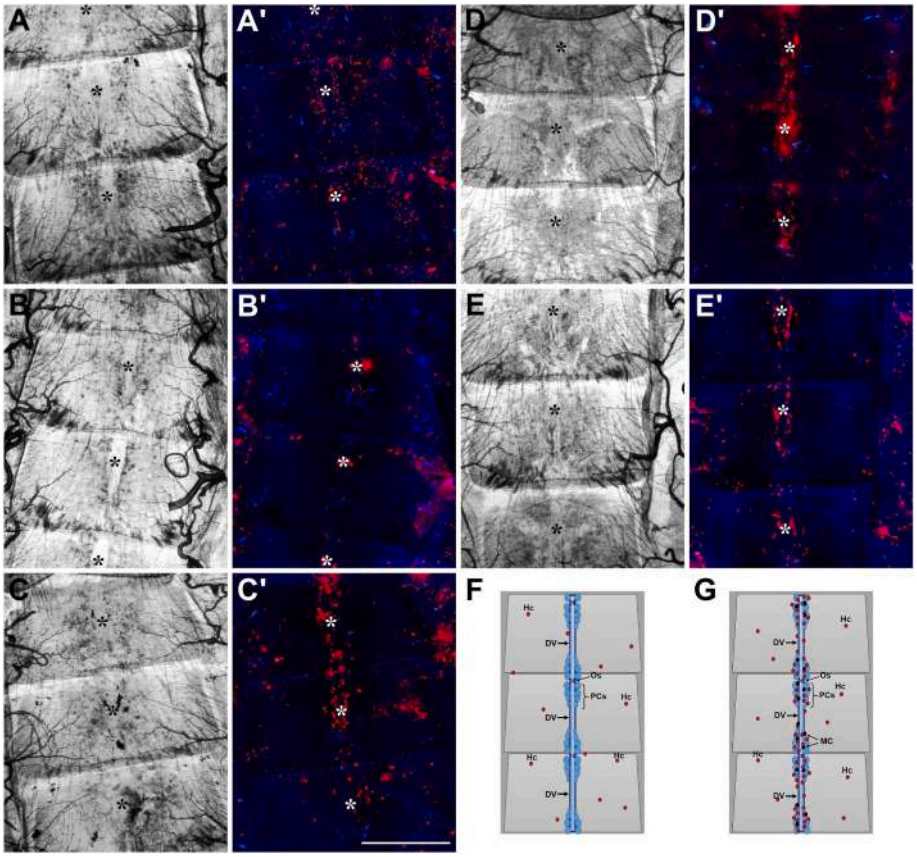

Fig. 3. Immune challenge induces hemocyte accumulation in the heart. Hemocytes accumulation in the heart was observed in whole abdomens from non-injected mosquitoes (A, A′), RPMI-injected (B, B′), and injected with *S. cerevisiae* (C, C′), *E. coli* (D, D′), *M. luteus* (E, E′). In red are hemocytes labeled with CM-Dil, nuclei from different cell types were stained with DAPI (in blue). Asterisks (*) indicate periostial regions. Scale bar = 500 μm. Drawing (F) represents hemocytes (Hc) distribution in non-treated whole mosquito abdomen. After an immune challenge (G), hemocytes and melanotic capsules (MC) are accumulated in periostial regions (Os) of the dorsal vessel (DV), where pericardial cells (PCs) are also located. All samples were obtained from female mosquitoes of 3 days-old at12 h post-injection. Images were obtained in an epifluorescence microscope and show representative areas from 5 samples to each condition. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

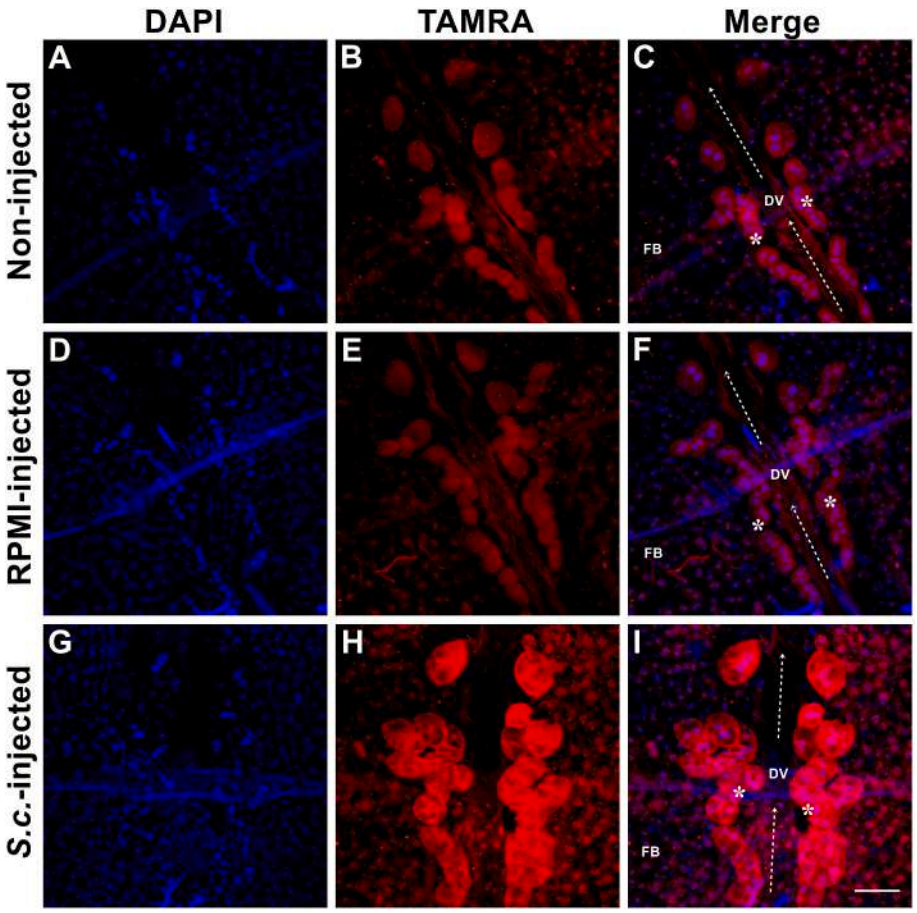

Fig. 4. Pericardial cells upregulate *Cec1* expression after an immune challenge. Transcripts of *Cec1* were detected by RNA FISH-STICs on abdomens from non-injected mosquitoes (A, B, C), RPMI-injected (D, E, F) or *S. cerevisiae*-injected mosquitoes (G, H, I). Nuclei are stained with DAPI (in blue), and transcripts of *Cec1* are detected by the probes tagged with TAMRA (in red). After the immune challenge, the *Cec1* transcripts increase in pericardial cells and fat body. In addition, pericardial cells from *S. cerevisiae*-injected showed an evident increase in size. Arrows are localized on fourth and fifth abdominal segments, pointing to anterior part of mosquito. (*) pericardial cells, (DV) dorsal vessel, (FB) fat body. Images were obtained in a confocal microscope, and show representative areas obtained from 5 samples to each condition. Scale bar (50 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

immune response.

4. Discussion

Mosquito-borne diseases are a worldwide public health problem. Since the most successful strategies are focused on vector control, it is important to understand the mechanisms that can limit pathogen transmission from mosquitoes to humans (Ghosh et al., 2000; Miller and Pierce, 2009; World Health Organization, 2017, 2020). In the last decades, researchers have focused primarily on the study of mechanisms that reduce parasite development in the midgut (Crompton et al., 2014; Sinden, 2015; Smith et al., 2014), however, while in midgut parasites multiplicate, leave the midgut and invade the mosquito's salivary gland (Douglas et al., 2015; Hillyer et al., 2007). During its journey, parasite must travel in and deal with hemolymph immune effectors such as hemocytes, antimicrobial peptides, phenol oxidase, etc. (Dimopoulos, 2003; Hillyer, 2010; Kumar et al., 2018; Moreno-García et al., 2014), where most of them are cleared before reaching the salivary glands (Hillyer et al., 2007). Surprisingly, even though most pathogens are eliminated in the hemocoel, the mechanisms involved remain unknown.

The heart is the most important organ of the mosquito circulatory system, distributing and regulating the hemolymph flow throughout the body (Hillyer and Pass, 2020). It has recently been discovered that *Plasmodium* sporozoites take advantage of the flow of hemolymph as a means of transport to invade the salivary glands (Hillyer et al., 2007). In addition, in *An. gambiae* during a bacterial infection, a specific population of hemocytes accumulates around the heart (periostial region) (Sigle and Hillyer, 2016). Interestingly, we observed *M. luteus* and *S. cerevisiae* injections stimulate the accumulation of hemocytes in the *An. albimanus* heart (Fig. 3). However, both *An. gambiae* and *An. albimanus* hemocytes primarily phagocytize small bacteria such as *E. coli*, whereas larger microorganisms, such as *M. luteus* bacteria and *S. cerevisiae* yeast, are mainly eliminated by melanization or lysis (Castillo et al., 2006; Hernández-Martínez et al., 1999, 2002; Hillyer et al., 2003; Sigle and Hillyer, 2016). Furthermore, in *An. gambiae*, sporozoites accumulate in periostial regions, but just a small number of them are phagocytosed by hemocytes, and some of them were observed partially fragmented, most likely by humoral effectors (King and Hillyer, 2012). Because we observed hemocyte accumulation in the *An. albimanus* heart (Fig. 3) it is possible that in a similar way as in *An. gambiae*, this accumulation facilitates the interaction of hemocytes with bacteria (Sigle and Hillyer, 2016), improving cellular and humoral immune responses.

The circulatory and immune systems of animals, including insects, are naturally linked (Yan and Hillyer, 2020). In *An. gambiae*, the accumulation of hemocytes in periostial regions during the interaction with pathogens, is regulated by the activation of IMD and JNK pathways that occurs in the heart with periostial hemocytes (Yan et al., 2022). Most likely, the activation of these two pathways is to control the expression of TEP1, TEP3, TEP4 (Yan and Hillyer, 2019) and Eater, which also plays a similar role in *Drosophila melanogaster* (Bretscher et al., 2015; Sigle and Hillyer, 2018). In *An. gambiae* expression of *Cec1* is regulated by IMD pathway (Meister et al., 2005), suggesting a possible synergic cooperation between hemocytes and heart cells (PCs), which reinforce the link of immune and circulatory systems. Moreover, in *D. melanogaster* PCs produce Reactive Oxygen Species (ROS) that activate IMD and the signaling cascade by Ask1, JNK and p38 that regulate the expression and releasing of cytokine Upd3 by PCs to the hemolymph (Gera et al., 2022). Once in the hemolymph, Upd3 modulates the fat body specific expression of the protein Pericardin (through the activation of JAK/STAT pathway), which is essential for cardiac function. Also, this protein acts as a scaffold, which allows hemocyte-heart interaction (Cevik et al., 2019), implying that PCs could be essential during the infection-induced

tripartite interaction (hemocytes/pathogens-heart). Also, *D. melanogaster* PCs have a critical function in maintaining immune homeostasis, through the removal of peptidoglycan and its subsequent degradation, most likely with a lysozyme (Troha et al., 2019). In addition to ROS, the nitric oxide generated by the Nitric Oxide Synthase (NOS) from heart-associated hemocytes, has been involved in the inhibition of the heart rate in *Baculum extradentatum* and *An. gambiae*, suggesting a strong dependence between immune and circulatory systems (Da Silva et al., 2012; Estévez-Lao et al., 2020). On the other hand, TEP1 was observed on PCs (Levashina et al., 2001), and we previously reported that *An. albimanus* heart express immune related genes such as IMD, cecropins and lysozymes (Hernández-Martínez et al., 2013b), and we recently confirmed PCs express the lysozyme c-1 (Cardoso-Jaime et al., 2021). These pieces of evidence indicate that PCs could play a variety of roles in the immune response.

Interestingly, PCs flank mosquito heart ostia, the same place where hemocytes are accumulated after an immune challenge (King and Hillyer, 2012; Sigle and Hillyer, 2016). Despite the fact that PCs are known for their filtering capabilities (Crossley, 1972; Fife et al., 1987), immunity markers such as STAT (Barillas-Mury et al., 1999), Serpin 10 (Danielli et al., 2003; Lycett et al., 2004), TEP-1 (Levashina et al., 2001), Sp22D, and Defensin (Danielli et al., 2000), have been found in *An. gambiae* PCs. We previously described the antimicrobial properties of *An. albimanus* PCs after an immune challenge (Hernández-Martínez et al., 2013b), which are related to some up-regulated immunity genes identified in a mosquito heart transcriptome (Martínez-Barnetche et al., 2012). In addition, we reported that one of these genes (*Lysozyme c-1*) is expressed by PCs in the mosquito heart (Cardoso-Jaime et al., 2021), indicating that PCs may play a role in the mosquito immune response, by secreting molecules that help to kill pathogens.

Antimicrobial peptides have a variety of roles in the immune response, and their participation during the parasite and arbovirus elimination has been documented (Chalk R. et al., 1995; Feng et al., 2020; Gwadz et al., 1989; Isaacs et al., 2011; Jaynes et al., 1988; Kim et al., 2004; Kokoza et al., 2010; Rodríguez et al., 1995; Xiao et al., 2014). *Hyalophora cecropia* cecropins were the first insect antimicrobial peptides described (Hultmark et al., 1980; Steiner et al., 1981). Since this first report, cecropins were identified in many other insects, including mosquitoes (Bulet and Stöcklin, 2005; De et al., 2018; Neafsey et al., 2015; Ponnuvel et al., 2010; Wang et al., 2010; Zheng and Zheng, 2002). Cecropin expression has been reported in midgut, salivary glands, Malpighian tubules, and other organs., however, the fat body is considered the primary organ producing cecropins (Dickinson et al., 1988; Lowenberger et al., 1999; Tzou et al., 2000). In the present study, we found that the mosquito heart express higher *Cec1* than the fat body (Fig. 1), yet the fat body represents greater tissue volume in the mosquito. Similar findings were reported in *M. sexta*, where the PCs complex (heart) expressed more cecropin than other tissues (Dickinson et al., 1988). This indicates that the heart is most likely one of the most important sites of cecropin synthesis.

Because the cecropins are involved in the immune response against Gram ± bacteria, yeast, and protozoan (Carboni et al., 2022; Chalk R. et al., 1995; DeLucca et al., 1997; Dong et al., 2020; Hultmark et al., 1980; Kajla et al., 2010; Lowenberger et al., 1999), we decided to evaluate *Cec1* expression in *An. albimanus* heart after an immune challenge with *M. luteus* (Gram + bacteria), *E. coli* (Gram – bacteria), and *S. cerevisiae* (yeast). We observed that *S. cerevisiae* and *M. luteus* induced the highest levels of expression, which is similar to previous findings in *An. gambiae* (Meister et al., 2005; Vizioli et al., 2000). *An. gambiae* has four cecropin genes (*Cec1-4*) (Kwon et al., 2021; Zheng and Zheng, 2002), three of which (*Cec1-3*) form a cluster, and *Cec1* and *Cec2* are regulated by the same promoter (Zheng and Zheng, 2002). In addition, the IMD pathway has been reported to regulate the expression of *Cec1-3*, *Gambicin 1*, *TEP1* and other immune genes (Blumberg et al., 2013; Meister et al., 2005), suggesting that additional genes to *Cec1* could be expressed in the mosquito heart. We opted to limit our analysis to *Cec1*, since it was one of the most upregulated genes in the transcriptomes of *An. albimanus* and An. gambiae heart (Hernández-Martínez et al., 2013b; Yan et al., 2022), and because *Cec1* can be involved in the resistance to *Plasmodium* infection (Isaacs et al., 2011; Kim et al., 2004; Kokoza et al., 2010). However, more research is needed to test whether other immunity-related genes are expressed in the mosquito heart.

Here, by RNA FISH-STIC (which is a sensitive and flexible approach for detecting both long and short RNA molecules, such as mRNAs molecules of AMPs) we show evidence that supports *Cec1* expression in PCs (Fig. 4), strongly indicating that PCs are responsible for *Cec1* expression in the mosquito heart. In addition, an interesting finding, was the fact that PCs increase in size after the immune challenge, which could be related to an increase in their metabolism and production of different molecules, including immune factors. In this regard, in *An. gambiae* significant differences in PC size have been reported between resistant and susceptible strains to *Plasmodium* infection, which was associated with increased production of reactive oxygen species and increased metabolism of PCs (Kumar et al., 2003). In the current work, we report for the first time the application of RNA FISH-STIC for the detection of a short mRNA (cecropin mRNA) in insect tissues. We propose that this tool can be used to detect *in situ* other short mRNA molecules in addition to AMPs, such as neuropeptides, ligands, cytokines, and other that are difficult to detect due to abundance or the size of the tissue in which they are expressed.

AMPs with fungicidal and antiparasitic properties, such as cecropins, can lyse pathogens (Durvasula et al., 1997; Gwadz et al., 1989; Jaynes et al., 1988; Kokoza et al., 2010; Rodríguez et al., 1995). We hypothesize that *Plasmodium* sporozoites close to PCs or circulating in the hemolymph may experience evident damage and even total lysis, due to a strong humoral immune response, in which possibly Cec1 may be involved. Some observations that partially support our hypothesis were reported by (King and Hillyer, 2012), nevertheless, they concluded that sporozoites were eliminated by a strong hemocyte-mediated cellular immune response. Although the participation of PCs was not considered in their work, our data support the idea that the PCs might be essential protagonists in the process of elimination of pathogens through humoral mechanisms, and that PCs can be part of a tripartite interaction between hemocytes and pathogens, secreting humoral factors that help to fight pathogens.

5. Conclusions

Our current findings contribute to a series of evidence which indicate that PCs have immunocompetent functions. PCs express Cec1 which can be altered differentially depending on the microbial challenge. This suggests that PCs probably have different signaling pathways that regulate their immune response. Based on the results of our study, we propose that PCs and hemocytes may be cooperating to perform a more efficient immune response against pathogens that cannot be eliminated solely by means of a cellular immune response.

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Author contribution

Conceptualization: S.H.M and V.C.J; Investigation: V.C.J, K.M.M., S. H.M; Methodology: V.C.J, S.H.M., K.M.M.; Formal Analysis: V.C.J., S.H. M., K.M.M., V.T.; Funding acquisition: S.H.M. and V.T.; Supervision: S. H.M. and V.T.; Writing: V.C.J., S.H.M., and V.T.; All authors reviewed the manuscripts.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2023.104745.

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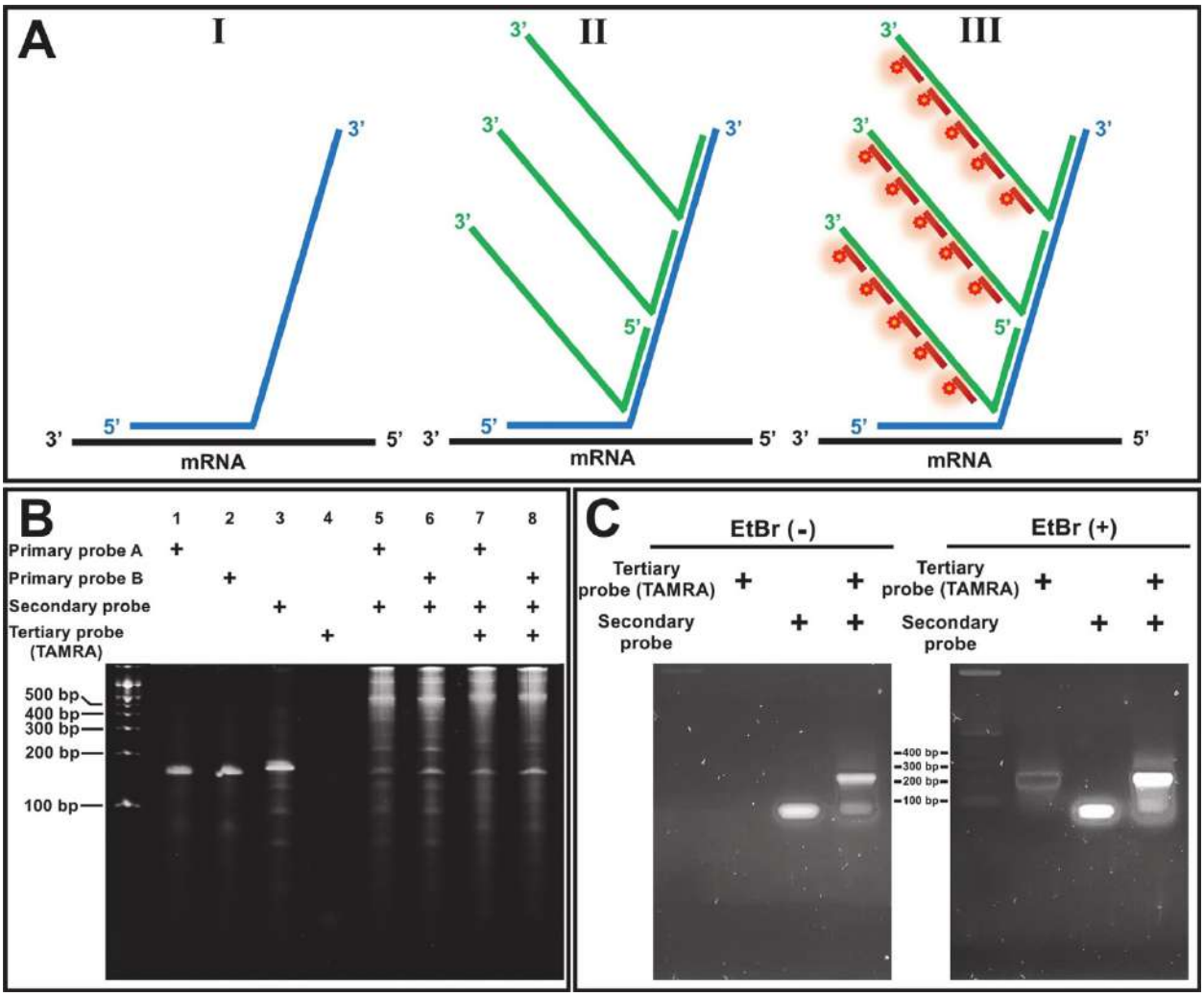

Fig. s1. RNA FISH-STICs. RNA FISH-STICs is an acid nucleic hybridization technique modified of conventional RNA-FISH, which uses three different probes that interact by themselves and with an mRNA target. (A-I) The primary probe (blue) has a complementary sequence that hybrids with the mRNA target, and the other sequence region have three identic sequences in tandem complementary to the secondary probe. (A-II) The secondary probe (green), has a complementary sequence to the primary probe, also contains 5 identic sequences in tandem complementary to tertiary probes. (A-III) The tertiary probe (red) sequence is only complementary to the secondary probe but contains a fluorescent tag (TAMRA), which allows the detection of probes-mRNA complex. To verify that probes used in the assay hybrid by themselves, equimolar quantities of each probe were previously incubated to interact by themselves, then mixes were run in a polyacrylamide gel (B). Each probe (only a probe) (1-4 lanes), mix of primary probes plus secondary probe (5, 6 lanes), mix of all probes (7, 8 lanes). The gel shows complex with different weights due to the probes formed different hybridization complex by themselves, suggesting that all sequences probes are correct and complementary. To verify the integrity of the TAMRA tag from the tertiary probe, the tertiary probe and a mix of tertiary probe plus secondary probe were run in an agarose

gel (C). Secondary probe (10 pM, lane 1), tertiary probe (50 pM, lane 2), mix of 10 pM secondary probe plus 50 pM tertiary probe (lane 3). The left gel in (C) is not stained with EtBr, but the tertiary probe and mix probes are observed due to the TAMRA tag from the tertiary probe is intact, also the secondary probe is observed only in the mix, indicating that secondary and tertiary probes are hybridized. The right gel in (C) is the same gel after staining with EtBr, where secondary probe (without tertiary probe) is observed.

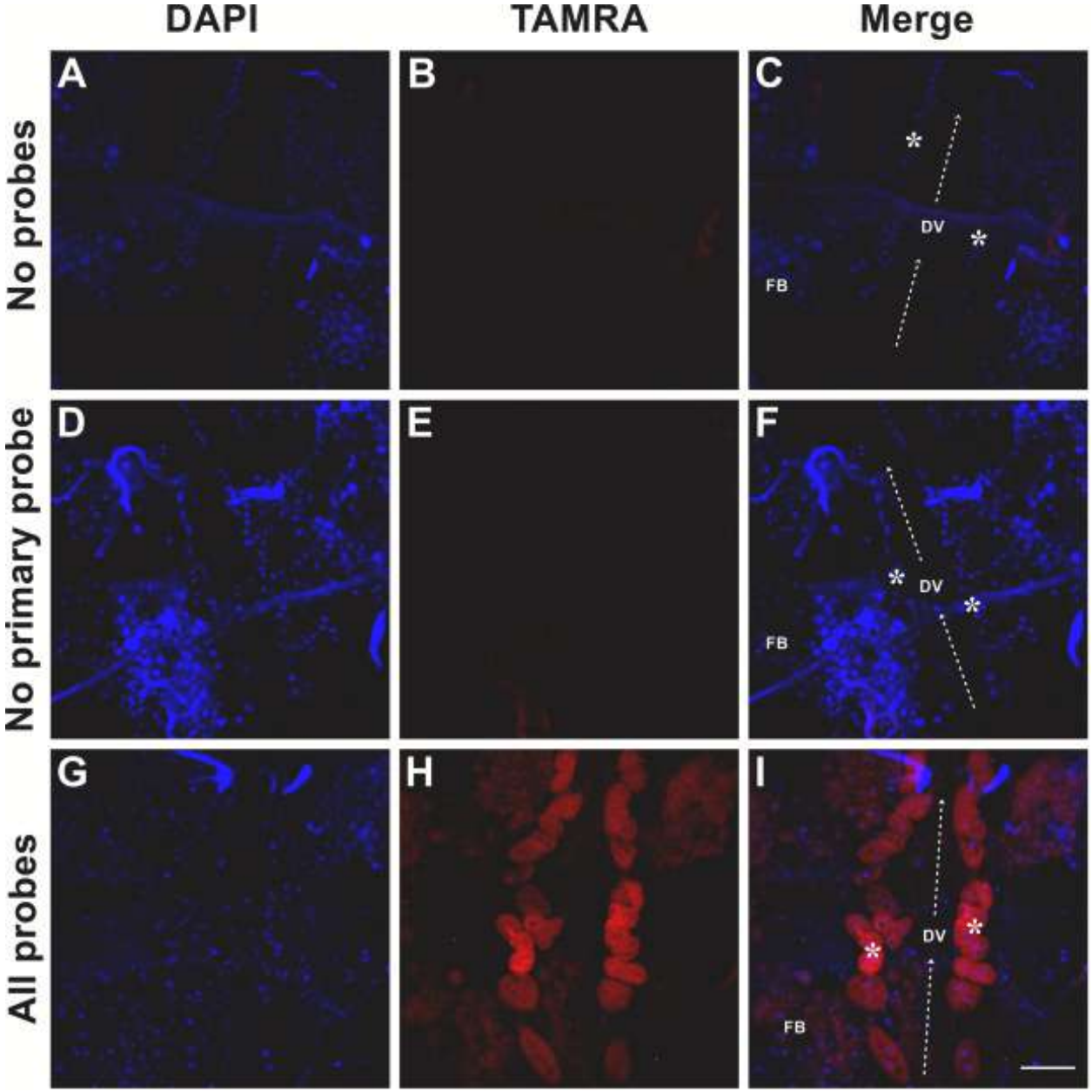

Fig. s2. *Cec1* mRNA detection by RNA FISH-STICs. *Cec1* mRNAs were detected by RNA FISH-STICs in mosquito abdomens. RNA FISH-STICs is a technique that uses three different probes, however, just the primary probes hybridize with mRNA target (*Cec1*). To demonstrate specificity of the assay were used the following controls: A control without probes (No probes: A, B, C), control without primary probes (No primary probes: D, E, F), RNA FISH-STICs complete using all the probes (All probes: G, H, I). In the control without primary probes signal was not observed, however, with all probes the signal was observed, which indicates that only the primary probes hybridize with the *Cec1* mRNA. Nuclei of different cell types of the heart are stained with DAPI (in

blue), in red the *Cec1* mRNAs detected by FISH-STICs probes tagged with TAMRA. (DV) dorsal vessel, (FB) fat body, (*) pericardial cells, dotted arrows indicate anterior direction of mosquito body. Images were obtained in a confocal microscope and show representative areas of 5 samples to each condition. Tridimensional images were obtained from z-stacks, but are represented in single 2D projection by maximum intensity projection. Scale bar = 200 μm.Fig. s2*Cec1* mRNA detection by RNA FISH-STICs. *Cec1* mRNAs were detected by RNA FISH-STICs in mosquito abdomens. RNA FISH-STICs is a technique that uses three different probes, however, just the primary probes hybridize with mRNA target (*Cec1*). To demonstrate specificity of the assay were used the following controls: A control without probes (No probes: A, B, C), control without primary probes (No primary probes: D, E, F), RNA FISH-STICs complete using all the probes (All probes: G, H, I). In the control without primary probes signal was not observed, however, with all probes the signal was observed, which indicates that only the primary probes hybridize with the *Cec1* mRNA. Nuclei of different cell types of the heart are stained with DAPI (in blue), in red the *Cec1* mRNAs detected by FISH-STICs probes tagged with TAMRA. (DV) dorsal vessel, (FB) fat body, (*) pericardial cells, dotted arrows indicate anterior direction of mosquito body. Images were obtained in a confocal microscope and show representative areas of 5 samples to each condition. Tridimensional images were obtained from z-stacks, but are represented in single 2D projection by maximum intensity projection. Scale bar = 200 μm.

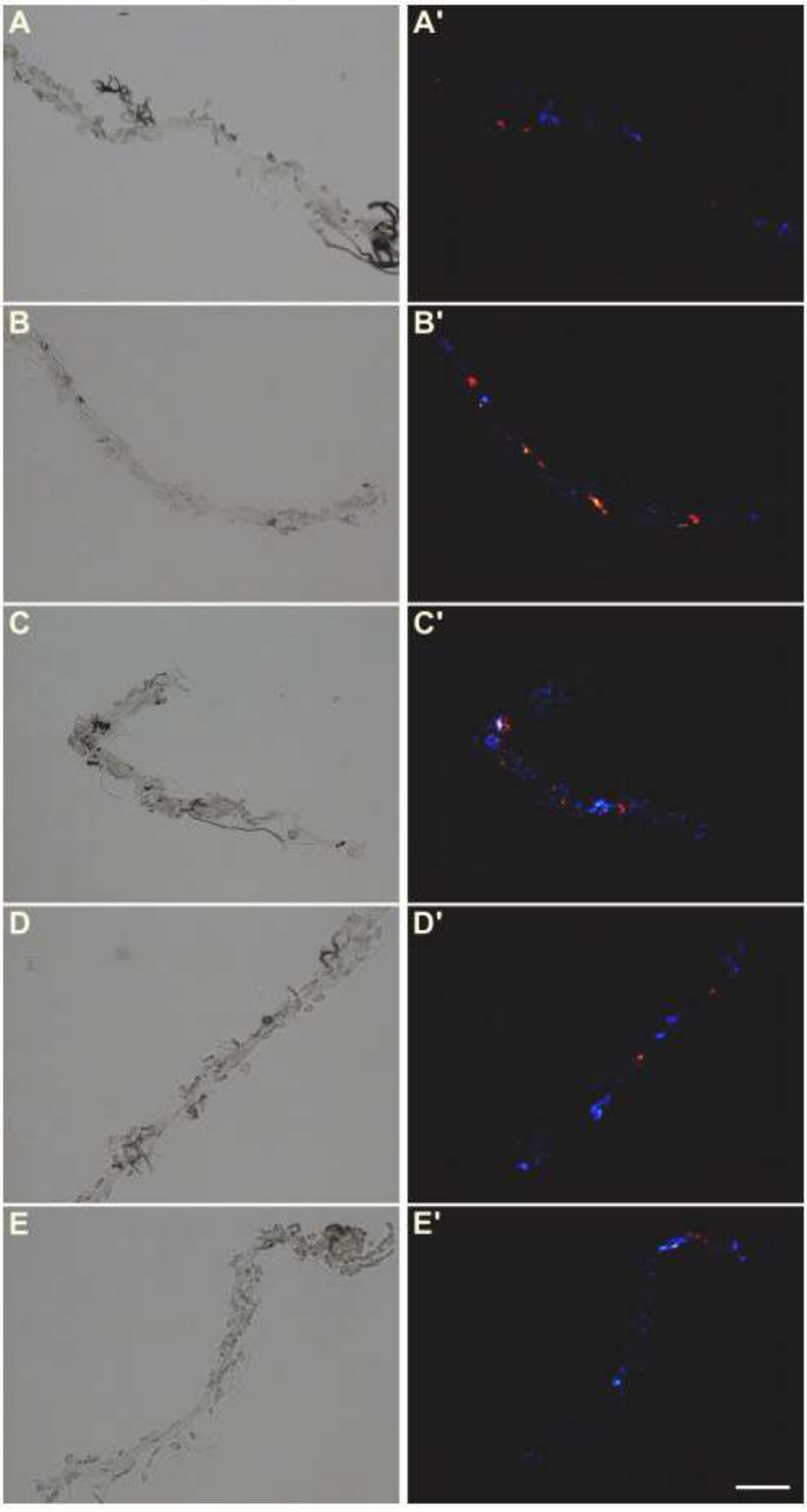
A
A'
B
B'
C
C'
D
D'
E
E'

Fig. s3. Isolated hearts have few hemocytes attached to them after washing. The hearts were obtained from female mosquitoes: non-injected (A, A'), RPMI-injected (B, B'), injected with *S. cerevisiae* (C, C'), *E. coli* (D, D') and *M. luteus* (E, E'). Nuclei from different cell types in hearts were stained with DAPI (in blue), and only hemocytes were stained by CM-Dil (in red). Hearts were obtained at 12 hour post-injected, then they were carefully washed three times whit PBS to remove most hemocytes attached to them. After washed, few hemocytes were observed on hearts, independently of treatment, indicating that all samples were enrichment with cell types coming from hearts. Images were obtained in an epifluorescence microscope and show representative areas from 5 samples to each condition. Scale bar = 200μm.

Supplementary material 1

RNA FISH-STICs probes sequences

The red highlight in primary probes (*Cec1*_Primary-probe1, and *Cec1*_Primary-probe2), indicates the sequences complementary to *Cec1* mRNA, and yellow highlight show repeat adapter sequences (each repeat sequence is delimitated by uppercase and lowercase letters) to the secondary probe (Secondary-probe).
The Yellow highlight in secondary probe indicates the complementary sequence to repeat adapter sequences from primary probes, green highlight shows the repeat adapter sequences (each repeat sequence are delimitated by uppercase and lowercase letters) to the tertiary probe.
Tertiary-probe sequence is complementary to repeat adapter sequences from secondary probe (green highlights). Additionally, tertiary probe contains a fluorescent Taq (TAMRA).

>*Cec1*_Primary-probe1
AGCAGGAGCGCAGCGACCACGACCAGGACCAGAACCTTGTTGAAGTTCATTCGTT
GGCCCCCGACCGTTACAGACTGTTCTCAGTtcgttggcccccgaccgttacagactgttctcagtTCGT
TGGCCCCCGACCGTTACAGACTGTTCTCAGT

>Cec1_Primary-probe2
CGTTAAACACGCGCTTCCCAGCTCCTTCGATCTTTTTGCCAAGCTTTTTGTCGTTG
GCCCCCGACCGTTACAGACTGTTCTCAGTtcgttggcccccgaccgttacagactgttctcagtTCGTT
GGCCCCCGACCGTTACAGACTGTTCTCAGT

>Secondary-probe
ACTGAGAACAGTCTGTAACGGTCGGGGGCCAACGAacgcgattgactaccagactatacgACGC
GATTGACTACCAGACTATACGacgcgattgactaccagactatacgACGCGATTGACTACCAGAC
TATACGacgcgattgactaccagactatacg

>/TAMRA/Terciary-probe
CGTATAGTCTGGTAGTCAATCGCGT