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RESEARCH ARTICLE

The *Aedes aegypti* peritrophic matrix controls arbovirus vector competence through HPx1, a heme–induced peroxidase

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Abstract

Aedes aegypti mosquitoes are the main vectors of arboviruses. The peritrophic matrix (PM) is an extracellular layer that surrounds the blood bolus. It acts as an immune barrier that prevents direct contact of bacteria with midgut epithelial cells during blood digestion. Here, we describe a heme-dependent peroxidase, hereafter referred to as heme peroxidase 1 (HPx1). HPx1 promotes PM assembly and antioxidant ability, modulating vector competence. Mechanistically, the heme presence in a blood meal induces HPx1 transcriptional activation mediated by the E75 transcription factor. HPx1 knockdown increases midgut reactive oxygen species (ROS) production by the DUOX NADPH oxidase. Elevated ROS levels reduce microbiota growth while enhancing epithelial mitosis, a response to tissue damage. However, simultaneous HPx1 and DUOX silencing was not able to rescue bacterial population growth, as explained by increased expression of antimicrobial peptides (AMPs), which occurred only after double knockdown. This result revealed hierarchical activation of ROS and AMPs to control microbiota. HPx1 knockdown produced a 100-fold decrease in Zika and dengue 2 midgut infection, demonstrating the essential role of the mosquito PM in the modulation of arbovirus vector competence. Our data show that the PM connects blood digestion to midgut immunological sensing of the microbiota and viral infections.

Author summary

Arboviruses transmitted by *Aedes aegypti* are a major public health threat. The peritrophic matrix (PM) is an extracellular layer that surrounds the blood bolus. It acts as an immune

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barrier that limits microbiota interaction with midgut epithelial cells. However, its usually assumed that the viral infection precedes the PM formation, which does not impact vector competence. We identified a PM-associated peroxidase that controls PM integrity and intestinal immunology and does so because it is controlled by a transcription factor that integrates inputs from dietary heme and blood meal triggered ecdysone signaling. Therefore, PM-driven intestinal homeostasis is pivotal to dengue and Zika infection, unveiling a new physiological role of this barrier for arboviral establishment in the mosquito.

Introduction

Mosquito-borne viruses are emerging as global threats to public health. Female mosquitos ingest infected blood from a host and transmit the virus to another host during the next blood-feeding. As the first insect tissue infected by the virus, the midgut is the initial barrier that the virus must overcome to establish itself in the mosquito [1]. Because blood digestion occurs in the midgut concomitantly with viral infection of epithelial cells, digestion-triggered physiological events have a major influence on the course of intestinal infection [2].

The peritrophic matrix (PM) in mosquitoes is a semi-permeable chitinous acellular layer secreted by intestinal cells after blood feeding. The PM completely envelopes the blood bolus, and its structure avoids direct contact of the digestive bolus with the midgut epithelia [3,4]. The PM is the site of deposition of most of the heme produced from blood hemoglobin hydrolysis, thus limiting exposure of the midgut cells to harmful concentrations of heme, a pro-oxidant molecule [5]. Extensive gut microbiota proliferation occurs in most hematophagous insects after a blood meal. Therefore, the PM is a barrier that limits interaction of the tissue with the intestinal microbiota [6–8], playing a role analogous to the mammalian intestinal mucous layer [8]. The PM is mainly composed of chitin and proteins, and correct assembly of this structure is crucial to its barrier function. Additionally, the PM is a barrier for parasites such as *Plasmodium*, *Trypanosoma brucei*, and *Leishmania major*, which must attach to or traverse the PM to complete their development in an insect vector [9–12].

There are several studies on the role of reactive oxygen species (ROS) and redox metabolism on the gut immune response to pathogens. In *Drosophila melanogaster*, ROS production by a dual oxidase enzyme (DUOX, an NADPH oxidase family member) is triggered by pathogenic bacteria. The self-inflicted oxidative damage arising from DUOX activation is prevented by hydrogen peroxide scavenging via an immune-regulated catalase (IRC) [13,14]. In *Anopheles gambiae*, *Plasmodium* ookinete midgut invasion triggers a complex epithelial response mediated by nitric oxide and hydrogen peroxide that is crucial to mount an effective mosquito antiplasmodial response [15]. Furthermore, an *Anopheles gambiae* strain genetically selected to be refractory to *Plasmodium* infection exhibits enhanced activation of JNK-mediated oxidative stress responses [16,17]. In *Aedes aegypti*, it has been proposed that the dengue NS1 viral protein decreases hydrogen peroxide levels, preventing an oxidative intestinal environment, which is an adverse condition for both dengue and Zika viral infection [18,19]. Catalase silencing in the *Aedes aegypti* gut reduces the dengue infection prevalence rate [20]. The ROS generation by DUOX plays a key role in modulating proliferation of the indigenous microbiota, growth of opportunistic pathogenic bacteria, and dengue virus infection [7,18,21].

Kumar et al. (2010) showed that heme peroxidase 15 (HPx15), also referred to as immunomodulatory peroxidase (IMPer), is expressed in the *A*. *gambiae* midgut and uses the hydrogen peroxide generated by DUOX as a substrate to crosslink proteins of the mucous layer in the ectoperitrophic space, limiting diffusion of immune elicitors from the gut microbiota and thus

preventing activation of midgut antimicrobial responses to commensal bacteria. IMPer silencing results in constant activation of epithelial immune responses against both bacteria and *Plasmodium* parasites [22]. A similar immune barrier role for the PM against parasite infection has also been shown in tsetse flies infected with *T. brucei* and sandflies infected with Leishmania [12,23]. Therefore, most of the studies on the PM of insect disease vectors have focused on its role as a barrier for parasites, but much less is known about the influence of PM on viral infections or its contribution to gut homeostasis and immune responses in *A. aegypti*.

Here, we show that HPx1, a heme peroxidase associated with the *A. aegypti* PM, has a dual role, acting in the PM assembly crucial for its barrier function and as an antioxidant hydrogen peroxide-detoxifying enzyme. This role of HPx1 in midgut physiology and immunity highlights that dietary heme is a signal that by triggering HPx1 expression and PM function, produces a homeostatic response that controls ROS and AMP immune effectors, microbiota expansion, and viral infection.

Results

Aedes aegypti PM detoxifies hydrogen peroxide

After a blood meal, the antioxidant capacity of the *Aedes aegypti* midgut is increased by expressing enzymes and low molecular weight radical scavengers [24]. These protective mechanisms are complemented by the capacity of the PM to sequester most of the heme produced during blood digestion, which has been proposed to be a preventive antioxidant defense, as heme is a pro-oxidant molecule [5,25]. Fig 1A shows that *A. aegypti* PM exhibited hydrogen peroxide detoxifying activity up to 24 h after blood-meal (ABM) followed by a sharp decrease at 36 h, close to the end of blood digestion. The specific activity of the PM hydrogen peroxide scavenging activity was comparable to the activity found in the midgut epithelia 24 h after blood-feeding, which is attributed to a canonical intracellular catalase (SUP1A). However, silencing of the "canonical" intracellular catalase (AAEL013407-RB) did not alter the PM's ability to detoxify hydrogen peroxide at 24 h after feeding (Fig 1B), contrasting with previous reports showing that silencing of this gene effectively decreased epithelial cellular catalase activity, therefore suggesting that the activity in the PM is not due to the midgut intracellular catalase (AAEL013407-RB) [20]. This hypothesis received support from the observation that the hydrogen peroxide decomposing activity of the PM and midgut epithelia showed distinct *in vitro* sensitivity to the classical catalase inhibitor amino triazole (SUP1B). Moreover, neither depletion of the native microbiota by antibiotic treatment (SUP1C) nor feeding the insect an artificial diet (cell-free meal) devoid of catalase (SUP1D) altered PM hydrogen peroxide detoxification, additionally excluding the hypothesis of an enzyme originating from the microbiota or host red blood cells.

HPx1-dependent hydrogen peroxide scavenging by the PM

We hypothesized that the observed PM hydrogen peroxide detoxifying activity should be attributed to another enzyme encoded by the mosquito genome. Peroxidases are a multigene family of enzymes and the genome of *A. aegypti*, as with most other organisms, has many peroxidases. Peroxidases are grouped in three large families: glutathione, heme, and thioredoxin peroxidases. As the PM is an extracellular structure, we initially searched for peroxidases with a predicted signal peptide. Interestingly, this search identified ten peroxidases, all of them belonging to the heme peroxidase family, which also includes the secreted peroxidases of *Anopheles gambiae* [22] and *Drosophila melanogaster* [14]. Phylogenetic analysis showed that *A. aegypti* heme peroxidase 1 (HPx1) is a close homolog of the peroxidase HPx15/IMPer that promotes the crosslink of extracellular proteins in the gut lumen of *A. gambiae* (Fig 1C). As

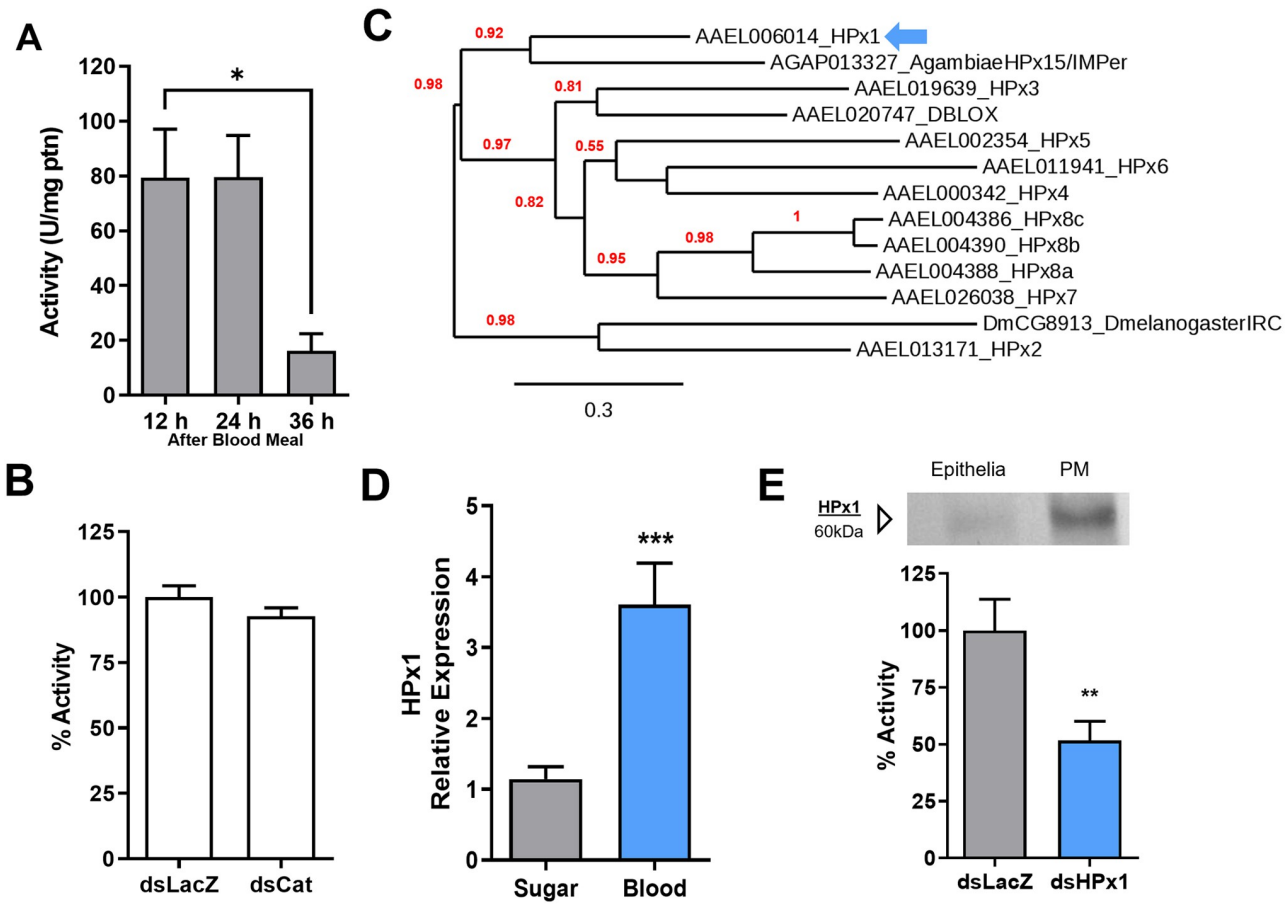

Fig 1. Hydrogen peroxide scavenging by the *Aedes aegypti* peritrophic matrix. A) PMs were dissected and pooled at different times after a blood meal (ABM), and their catalase specific activity was measured (12 h n = 5, 24 h n = 6; 36 h n = 5 pools of 5 PM). B) Catalase activity of PMs dissected from control (dsLacZ-injected) and catalase (AAEL013407-RB)-knockdown insects at 24 h AMB (dsLacZ n = 9; dsCat n = 9). C) Phylogenetic tree of heme peroxidases from *Aedes aegypti* (AAEL), *Anopheles gambiae*, and *Drosophila melanogaster*. Maximum likelihood analysis was performed, and the numbers in each branch represent bootstraps. (D) HPx1 expression in midguts at 24 h ABM relative to the sugar-fed control (Sugar n = 11; Blood n = 11). (E) Western blot of the HPx1 protein in 20 μg of gut epithelia and PM extracts at 18 h ABM and catalase activity of PMs dissected from control and HPx1-knockdown insects (dsLacZ n = 8; dsHPx1 n = 13). The full western blot membrane is shown in S1E Fig. Data are the mean +/- SEM. $^{**}p > 0.005$, $^{***}p < 0.001$ for the T test for D and E.

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the HPx15/IMPer from *A. gambiae* was shown to be secreted by the midgut epithelia, we used the presence of a secretion signal peptide as an additional feature to indicate HPx1 as the *A. aegypti* PM enzyme responsible for decomposing hydrogen peroxide. Fig 1D shows that the blood meal induced HPx1 gene expression in the gut. Moreover, western blotting showed that most of the HPx1 in the midgut was bound to the PM, with a minor fraction being associated with the epithelia (Fig 1E), and RNAi silencing of HPx1 expression significantly decreased hydrogen peroxide detoxification by the PM (Fig 1E; HPx1 silencing efficiency: SUP1F).

The E75 transcription factor mediates heme-induced HPx1 midgut expression

A blood meal triggers large changes in the gene expression pattern of *A. aegypti* and, among several factors, the heme released upon hemoglobin proteolysis acts as a pleiotropic modulator of transcription [26]. Feeding the insects with SBM with or without heme revealed that

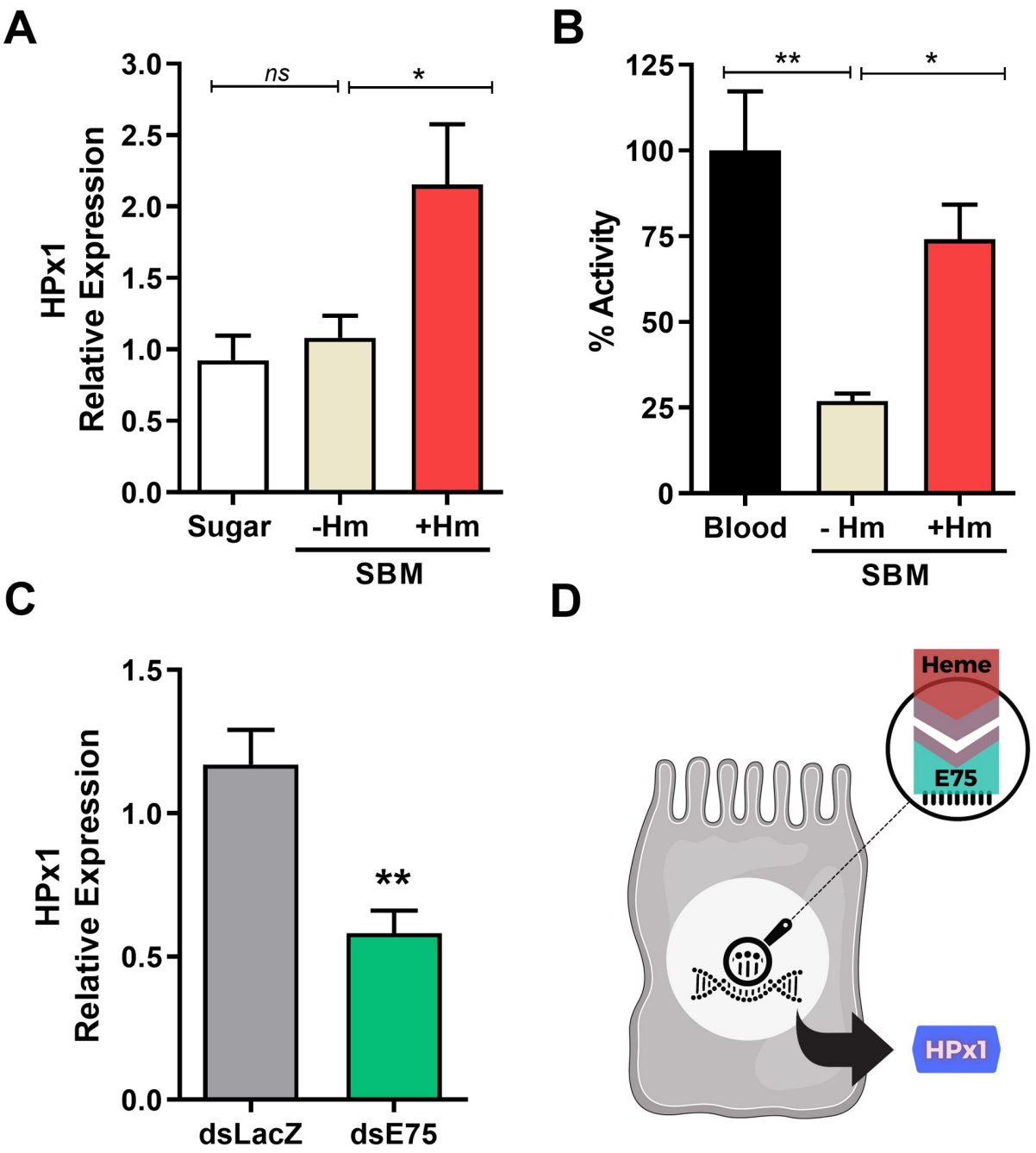

Fig 2. HPx1 expression is controlled by dietary heme and the E75 transcription factor. A) HPx1 expression in the midgut of sugar-fed or at 24 h after SBM feeding (without heme or supplemented with 50 μM of heme) (Sugar n = 5; -Hm n = 14; +Hm n = 14). B) Catalytic activity of PMs from mosquitoes fed different diets at 24 h postfeeding (Blood n = 3 pools of 10 PM each; -Hm n = 4 pools of 10 PM each; +Hm n = 4 pools of 10 PM each). C) HPx1 expression in midguts from control (dsLacZ) and dsE75-injected mosquitoes at 24 h ABM (dsLacZ n = 6; dsE75 n = 7). D) Schematic model of molecular signaling for HPx1 expression in the mosquito midgut. $^{*}p<0.05$, $^{**}p<0.005$, ns = not significant. Data are the mean +/- SEM. One-way ANOVA with Dunnett's post-test for A and B and the T test for C.

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heme significantly regulated HPx1 gene expression (Fig 2A). Accordingly, lower hydrogen peroxide decomposition activity was observed in the PM secreted by females fed SBM without heme compared to the blood-fed insects, a phenotype rescued by heme supplementation (Fig 2B). *In silico* analysis of the HPx1 promoter gene region revealed putative binding sites

for E75, a hormone-responsive transcription factor that functions as a heme and redox sensor (SUP2A) [27,28]. E75 knockdown significantly reduced HPx1 gene expression in the midgut after blood feeding, suggesting a molecular mechanism for triggering HPx1 by heme (Fig 2C; E75 silencing efficiency: SUP2B). Proliferation of the gut microbiota in response to blood feeding is known to induce expression of several genes in the midgut. However, neither microbiota depletion by oral administration of antibiotics (aseptic) nor reintroduction of a bacterial species (*Enterobacter cloacae*) into antibiotic-treated mosquitoes affected HPx1 gene expression (SUP2C). Thus, we propose the molecular signaling model shown in Fig 2D.

HPx1 contributes to PM assembly and regulation of the gut bacterial population

The PM is a semipermeable matrix that controls the traffic of molecules between the intestinal lumen and the epithelia, and its correct assembly is essential to fulfilling its barrier function. The PM morphology (observed by the staining of chitin fibers present in the intestinal lumen) was not heavily compromised by the HPx1 silencing (SUP 3C). Fig 3A shows that, when fed fluorescent dextran particles, different from silenced insects, control mosquitoes retained the polymer on the gut luminal side, a proxy of the normal barrier function of the PM. In contrast, HPx1-silenced mosquitoes presented strong fluorescence in the epithelial layer, suggesting a role for HPx1 in the proper assembly of the PM, as its permeability barrier function was compromised by HPx1 silencing. It has been proposed that the *A*. *gambiae* IMPer mediates a protein crosslinking by dityrosine bound, which maintains the PM barrier structure. Silencing HPx1 also reduces the dityrosine residues in the gut epithelia in *A*. *aegypti* (SUP3A-B). As this alteration in permeability might expose the epithelium to bacterial elicitors from the proliferative microbiota, we evaluated ROS production, known as an antimicrobial defense. Fig 3B and 3B' show that HPx1 silencing increased ROS levels in the midgut. The increase in ROS in HPx1-silenced mosquitoes was due to increased exposure of the gut epithelia to either the microbiota or microbiota-derived immune elicitors, once oral administration of the antibiotics prevented the increase in the ROS levels, as observed in HPx1-silenced mosquitos (Fig 3C). Exposure to damage signals and elevated ROS has been shown to activate intestinal stem cell mitosis (30). Indeed, HPx1 silencing increased phosphorylated H3-histone levels (Fig 3D) in midgut epithelial cells, indicative of mitotic activity and suggestive of epithelial remodeling in response to oxidative imbalance. This highlights the key role of HPx1 in tissue homeostasis. The native midgut bacterial load was significantly reduced after HPx1 silencing (Fig 3E). However, this was not due to the activation of canonical immune signaling pathways, as the expression of two antimicrobial peptides, Attacin and Cecropin G, was not significantly different from dsLacZ controls (Fig 3F). Interestingly, the bacterial sensor PGRP-LB decreased its expression as the total amount of microbiota was also reduced (Fig 3F). Overall, it highly suggests that ROS levels control the intestinal microbiota proliferation.

HPx1 and DUOX coordinate intestinal immunity

NADPH-oxidases are a family of ROS-producing enzymes related to the immune system. Members of the dual oxidase (DUOX) group have been shown to play an essential role against bacterial challenge in the insect intestinal environment [7,13,22]. Silencing HPx1 alone significantly increased ROS levels (Fig 4A). In contrast, ROS levels were similar to those of dsLacZ controls in females in which HPx1 and DUOX were co-silenced (Fig 4A and 4B), indicating that DUOX activity is the source of ROS when HPx1 is silenced. Unexpectedly, despite

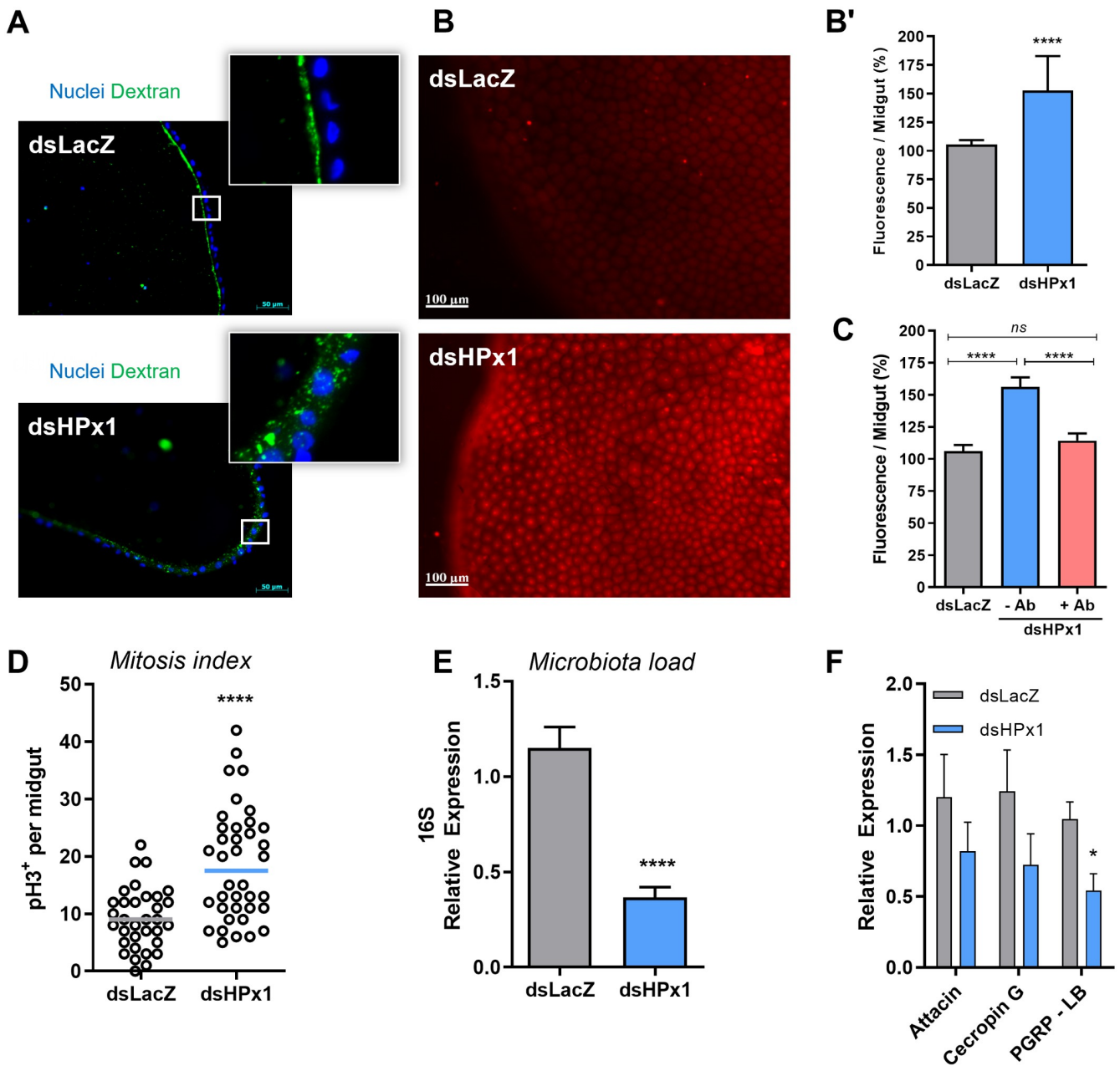

Fig 3. The role of HPx1 in PM assembly. A) Midgut transverse slices at 18 h ABM supplemented with dextran. Green: dextran—FITC. Blue: DAPI nuclear staining. Insets highlight dextran localization. B) Representative images of ROS levels measured by DHE oxidation in individual midguts at 18 h ABM. B') Quantitative analysis of the fluorescence intensity of oxidized DHE (dsLacZ, n = 35; dsHPx1, n = 39). C) Quantitative analysis of the fluorescence intensity of oxidized DHE from individual midguts at 18 h ABM (dsLacZ, n = 32; dsHPx1—Ab, n = 34; dsHPx1 + Ab, n = 27). D) Mitosis index in the mosquito midgut at 18 h ABM (phospho-histone H3) (dsLacZ n = 33; dsHPx1 n = 38). E) The intestinal microbiota load analyzed through eubacterial ribosomal 16S gene expression by qPCR at 24 h ABM (dsLacZ n = 6; dsHPx1 n = 7). F) Immune-related gene expression upon HPx1 silencing at 24 h ABM by qPCR (dsLacZ n = 8; dsHPx1 n = 7). $^{*}p<0.05$, $^{****}p<0.0001$, ns = not significant. Data are the mean +/- SEM. The T test for B', D, E and F, and one-way ANOVA with Tukey's posttest for C.

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lowered ROS levels in double-silenced mosquitoes, bacterial levels remained reduced (Fig 4C). This antibacterial response appears to be mediated by activation of canonical immune signaling pathways, as evidenced by increased expression of antimicrobial peptides and PGRP-LB in HPx1/DUOX co-silenced females (Fig 4D–4F).

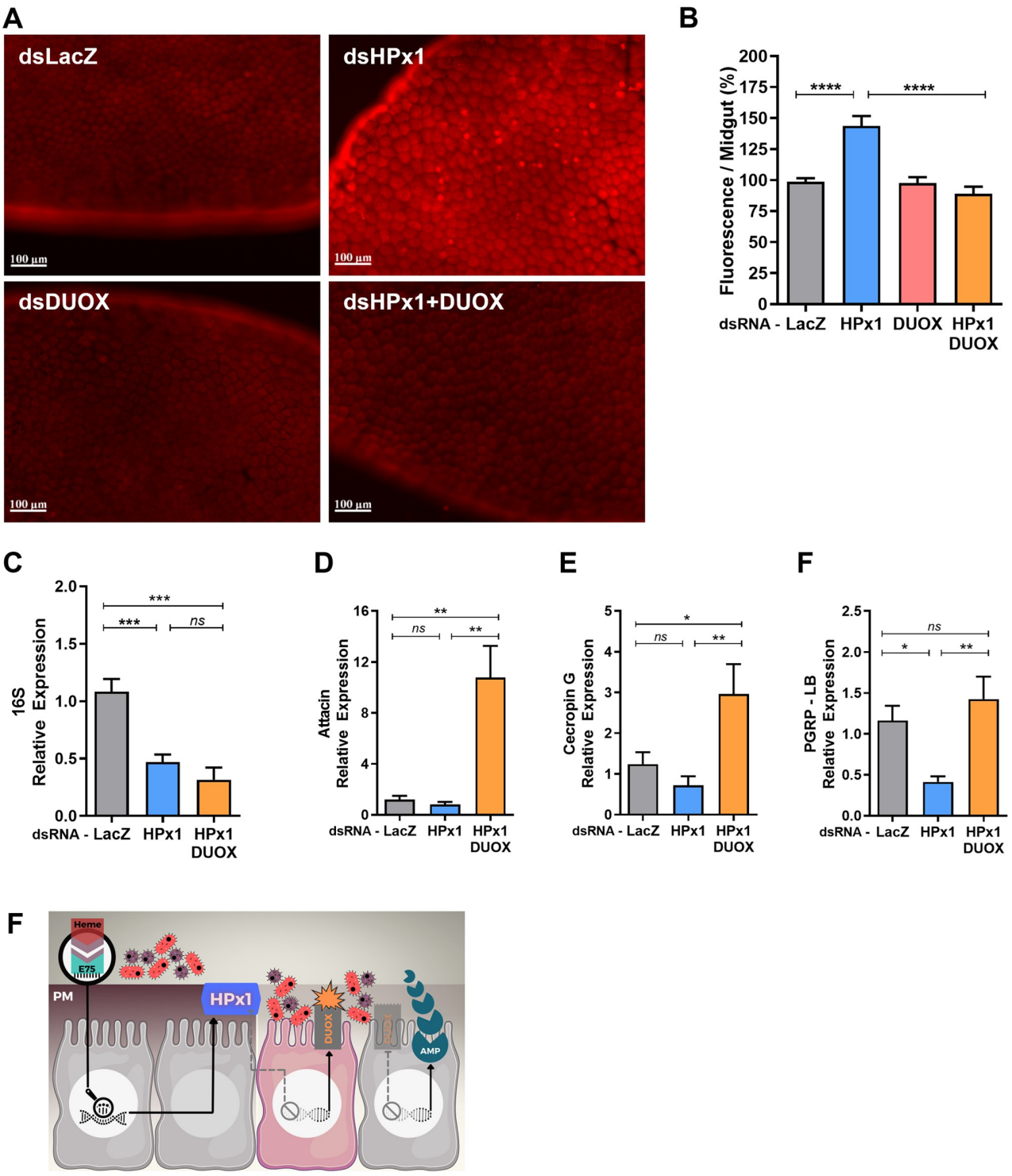

Fig 4. DUOX activation upon HPx1 silencing and hierarchical mode of activation of innate immunity in the mosquito midgut. A) Representative images of ROS levels measured by DHE oxidation in individual midguts at 18 h ABM. B) Quantitative analysis of the fluorescence intensity of oxidized DHE (dsLacZ, n = 38; dsHPx1, n = 43; dsDUOX, n = 21; dsHPx1+DUOX, n = 31). C) The intestinal microbiota load analyzed through eubacterial ribosomal 16S gene expression by qPCR at 24 h ABM (dsLacZ n = 6; dsHPx1 n = 6; dsHPx1+DUOX n = 4). D-F) Immune-related gene expression upon HPx1 silencing at 24 h ABM by qPCR (at least n = 5 for each condition). F) Schematic panel of intestinal immune activation showing that the PM integrity mediated by HPx1 activity isolates the gut microbiota, and once this integrity is lost, DUOX and antimicrobial peptides (AMPs) are activated. $^{**}p<0.005$, $^{***}p<0.001$, $^{****}p<0.0001$, ns = not significant. Data are the mean +/- SEM. One-way ANOVA with Tukey's post-test for A', B, and C.

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Intestinal homeostasis impacts Zika and dengue virus infection

The midgut epithelium is the first tissue a virus must invade to establish a successful infection. By modulating diffusion of immune elicitors, HPx1 is crucial to gut homeostasis, allowing the proliferation of the gut microbiota without triggering an immune response, which led us to investigate HPx1 role in arbovirus infection. Dengue infection prevalence was reduced in HPx1-silenced females, while the infection intensity was not affected (Fig 5A). On the other hand, Zika (ZIKV) infection intensity was reduced upon HPx1 silencing without affecting the infection prevalence (Fig 5B). This result suggests that DENV and ZIKV infection are differentially regulated by the HPx1 activity. As microbiota-derived elicitors activate ROS generation by the gut epithelia, we tested whether HPx1 silencing in mosquitoes treated with antibiotics to reduce microbiota levels would still impact gut infection. Fig 5B shows that the antibiotic treatment followed by the HPx1 silencing rescued the infection phenotype, confirming that microbiota-derived factors trigger a viral restriction response in the gut. Addressing the PM role by silencing the enzyme chitin synthase, responsible for the synthesis of a canonical PM structural component, affects the DENV infection in a way almost identical to what was observed in HPx1-silenced insects (SUP 4).

Discussion

The *Aedes aegypti* PM is an acellular layer that surrounds the blood bolus throughout the digestion process and limits direct contact of the epithelium with the midgut content and the intestinal microbiota, which undergoes massive proliferation upon blood feeding. In female mosquitoes, PM formation occurs in response to ingestion of a blood meal, following a time course that is finely coordinated with the pace of blood digestion. However, the signaling pathways that trigger PM secretion in adult mosquito females have not been elucidated nor has the impact of this structure on viral infection. Here, we characterize an intestinal secreted peroxidase (HPx1) that functions in PM assembly, contributing to its barrier function and promoting microbiota growth by preventing an antimicrobial response. This PM function has a permissive role for viral replication in the mosquito gut, thus constituting a novel determinant of vector competence. Importantly, dietary heme triggers HPx1 gene expression using the heme-dependent transcription factor E75, allowing synchronization of PM maturation with blood digestion by sensing the free heme released as hemoglobin is digested.

Similar to all blood-feeding organisms, mosquitoes face an oxidative challenge due to large amounts of heme—a pro-oxidant molecule—released by hemoglobin degradation. Therefore, preventing oxidative damage through ROS detoxification is a hallmark of their physiology [24]. HPx1 mediates a novel mechanism to promote redox balance in the *Aedes* midgut through its hydrogen peroxide scavenging activity and by modulating PM barrier function. We also show that HPx1 allows proliferation of the gut microbiota without activating a DUOX-mediated oxidative burst by limiting exposure of gut epithelial cells to microbial immune elicitors. We have previously shown that *Aedes aegypti* catalase (AAEL013407-RB), the main intracellular hydrogen peroxide scavenger, is induced in the blood-fed midgut of females [20]. Here, we demonstrate that HPx1 contributes to the overall peroxide scavenging capacity of the gut in a way that is independent of epithelial intracellular catalase but at comparable activity levels (SUP 1A). Although peroxidases are less efficient in decomposing hydrogen peroxide than catalases, it is known that some heme peroxidases also have high catalase activity [29,30].

A. gambiae IMPer/HPx15 belongs to anopheline-specific expansion of the heme peroxidase family [31] nested in the same branch of the peroxidase family of *A. aegypti* HPx1 and immune-regulated catalase (IRC; CG8913) of *D. melanogaster* [14,32,33]. However, *Drosophila*

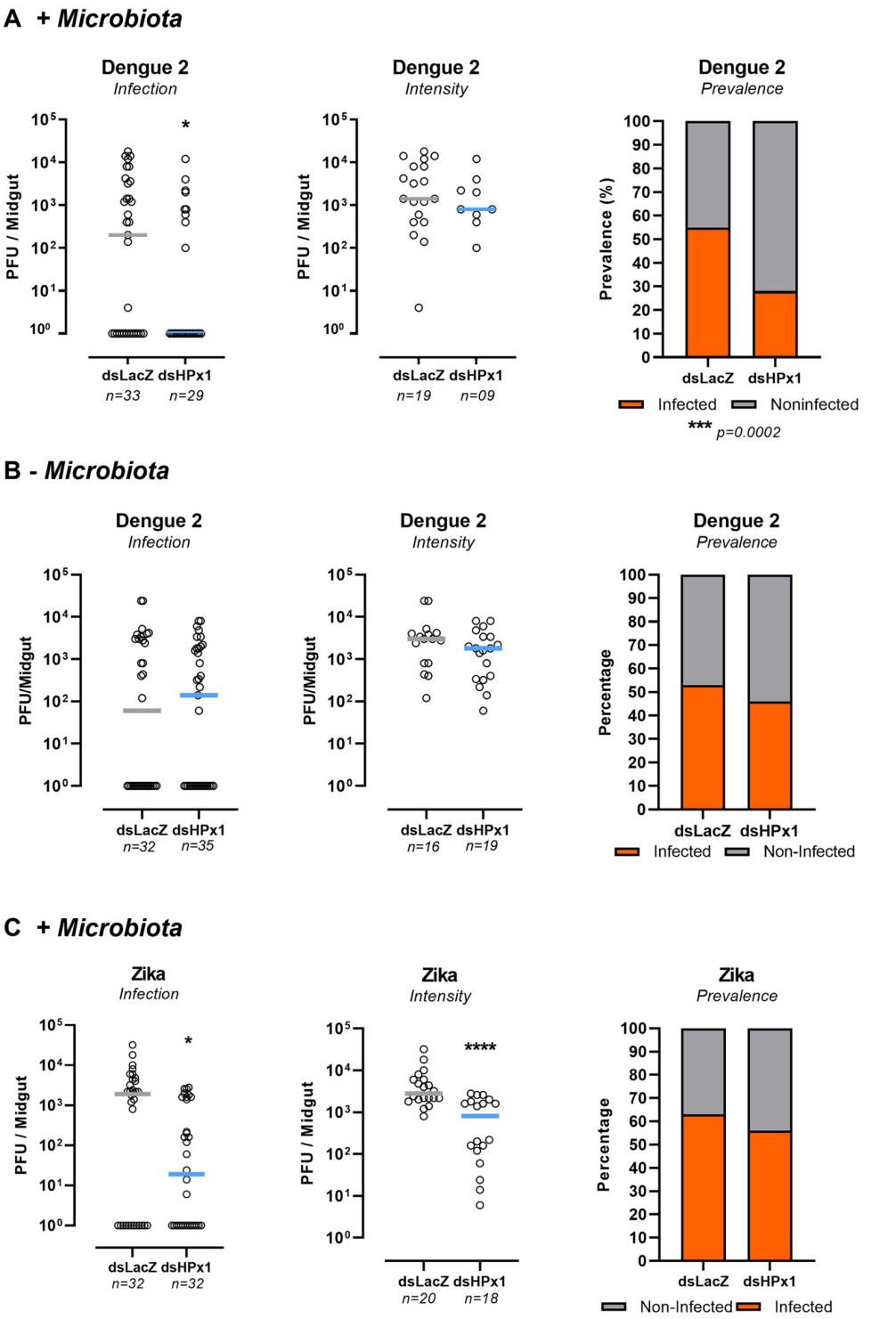

Fig 5. Viral infection in HPx1-silenced insects. A) Dengue 2 titers in midguts 7 days postinfection. B) Mosquitoes were treated with anitbiotics in sugar solution 3 days prior of DENV2 infection. Titers in midguts at 7 days postinfection. C) Zika titers in midguts at 4 days postinfection. Viral titers were assessed by the plaque assay. Each dot represents an individual mosquito gut, and bars indicate the median. * $p < 0.05$; Mann–Whitney test for A and B. The prevalence statistical analysis was performed by the Chi-square test followed by Fisher's exact test.

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IRC has a conserved heme peroxidase domain structure (Pfam PF03098) but lacks a catalase domain (Pfam PF00199), despite having a high hydrogen peroxide dismutation activity [14], which is a feature of catalases but uncommon among peroxidases. Interestingly, IMPer/HPx15 of *Anopheles* is also expressed in the female reproductive tract, induced by the ecdysone transferred along with the sperm during insemination, due to ecdysone-responsive elements present in the promoter region [34]. Ecdysone-responsive element sequences close to the *Aedes* HPx1 gene have been identified *in silico* [35]. Therefore, *Aedes* HPx1, IRC, and AgIMPer are secreted enzymes that modulate interactions of the midgut with commensal and pathogenic bacteria. Nevertheless, their role in the biology of the reproductive organs has not been well established. Although our data reveal that HPx1, IRC, and IMPer share sequence and functional homology, it is not possible at present to speculate which roles are ancestral and which were acquired secondarily during the evolution of dipterans.

A. *gambiae* IMPer has been proposed to crosslink external matrix proteins by forming dityrosine bridges, reducing the accessibility of microbial elicitors to the intestinal cells [22]. In *Drosophila*, however, a transglutaminase enzyme crosslinks PM proteins, also protecting the midgut epithelia from damage [36,37]. In this study, we show that HPx1 associates with the PM and modulates gut permeability in *A*. *aegypti* (Fig 4) and promotes protein crosslink by dityrosine bounds (SUP 3A-B). When the PM permeability was compromised by HPx1 silencing, we observed immediate responses from the epithelium that increased ROS levels, which was attributed mainly to DUOX activation by microbial elicitors. The role of PM in preventing elevated ROS production in the gut epithelium was also observed when the PM was compromised by the chitin inhibitor diflubenzuron administered in a blood meal for *A*. *aegypti* females [38]. A simple hypothesis to explain how elevated ROS might lower virus infection is that they directly attack the virus. However, arbovirus infection of midgut cells is thought to occur early during digestion, several hours before proliferation of microbiota occurs and before the PM is secreted [39]. Therefore, it is unlikely that extracellular ROS produced in response to bacterial elicitors in HPx1-silenced insects would directly attack virus particles that will be localized intracellularly when these molecules start to increase in the lumen. In general, cellular antiviral mechanisms are most plausibly responsible for hampering viral infection upon HPx1 silencing. Among these possible mechanisms, the elevated extracellular ROS levels derived from DUOX activation are indeed sensed by the gut as a danger signal, evoking a tissue-repairing response the hallmark of which is stem cell proliferation (Fig 4), a homeostatic response coupled to cell death in response to insult and damage. Interestingly, Taracena et al. proposed that different degrees of resistance to infection among mosquito strains are related to different capacities to promote a rapid increase in stem cell proliferation; hence, faster cell death, followed by cell renewal from stem cell activation, is a process that reduces viral infection [38]. One could hypothesize that this mechanism is responsible for the decrease in ZIKV and DENV infection promoted by HPx1 silencing.

In mammals, the mucus layer allows commensal bacteria from the gut microbiota to thrive without eliciting microbicidal immune responses by the intestinal mucosa. In other words, the mammalian mucus layer acts as a physical barrier that leads to "immunological ignorance" by preventing a state of constant immune activation and chronic inflammation of the intestine in response to immune elicitors from the normal microbiota [40–42]. In mosquitos, the PM is secreted when the microbiota peaks in number to approximately 100–1000 times the population found before a blood meal [7], representing a potential massive immune challenge to this tissue. Hixson et al. suggested that immune tolerance to the indigenous microbiota might be mediated by high expression of caudal and PGRP genes, leading to low expression of antimicrobial peptides in epithelial cells from the posterior gut [43]. Here, we highlight the role of PM-associated HPx1 in limiting exposure of the epithelium to immune elicitors from the

expanded microbiota observed postfeeding. Before blood feeding, the PM is absent, and bacteria interact with the epithelium, leading to ROS generation and damage-induced repair [7,38]. When the first line of immediate response to immune elicitors (redox mediated) is further prevented by simultaneous DUOX silencing, a reaction is activated (antibacterial peptides expression) to limit microbial growth. These data suggest hierarchal immune activation in the *A. aegypti* midgut fundamentally orchestrated by the PM integrity. This is similar to what was reported for *A. gambiae*, whereby bacterial elicitors lead to DUOX-mediated activation of IMPer, which promotes cross-linking of extracellular proteins [22]. However, in this report, an epithelial cell mucous layer (and not the PM) was indicated as the site of action of the peroxidase. Regarding the mode of activation of this pathway, the findings shown herein are also fairly similar to those for *Drosophila*, with ROS produced by DUOX being primarily triggered by bacterial pathogens, and the IMD pathway induces antimicrobial peptide production upon activation failure [21,43–45].

Traditionally, immunology has focused on how hosts eliminate pathogens while fighting infections, but in the last decade, there has been a growing interest in how hosts endure infection by utilizing disease tolerance, including diminishing both the direct damage caused by the pathogen and the self-inflicted damage due to the host immune reaction directed at the elimination of the pathogen. In this study, we showed that PM-associated HPx1 is pivotal to maintaining gut immune homeostasis, acting as a tolerance mechanism that prevents responses to the microbiota and, consequently, to the viral infection. This conclusion was reinforced by the fact that antibiotic treatment prevented the effect of HPx1-silencing on virus infection. The chitin synthase silencing, used as an approach to disrupting PM formation by an independent mechanism, recapitulates the HPx1 silencing impact on dengue 2 infection and stretches the claim that PM integrity is essential to gut homeostasis and has a permissive role for viral infection.

Additionally, our data revealed that, although belonging to the same family, ZIKV and DENV infection outcomes were distinct when HPx1 was silenced, once only the infection intensity was affected in the case of ZIKV, in contrast to a prevalence effect when DENV was evaluated. It has been reported that ZIKV and DENV do not necessarily exploit the same transcriptional response in the mosquito gut, indicating they play different roles in modulating the immune and physiological responses [46]. In this way, the HPx1 can have distinct roles toward each different viruses.

Together, our results indicate that the *A. aegypti* PM supports midgut homeostasis during blood digestion. Heme derived from blood hemoglobin digestion regulates expression of HPx1, an enzyme that has a central role in the assembly of a fully functional PM, through the heme-sensitive E75 transcription factor. Thus, HPx1 maintains immunological ignorance of the midgut epithelia toward the microbiota, allowing a state of microbiota and viral tolerance and preventing tissue damage.

Materials and methods

Ethics statement

All the animal care and experimental protocols were conducted following the guidelines of the institutional care and use committee (Comissão de Ética no Uso de Animais, CEUA-UFRJ) and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. The protocols were approved under the registry #CEUA-UFRJ 149/19 for rabbit use and 075/18 for mice immunization and antiserum production. The animal facility technicians at the Instituto de Bioquímica Médica Leopoldo de Meis (UFRJ) carried out all aspects related to rabbit and mice husbandry under strict guidelines to ensure humane animal handling.

Mosquitos

The *Aedes aegypti* females (Red-Eye strain) used in this study were raised in an insectary of Universidade Federal do Rio de Janeiro. Approximately 200 larvae were reared in water-containing trays and fed dog chow. Pupae were transferred to plastic cages, and adults were fed *ad libitum* with 10% sucrose solution in cotton pads. The insects were kept in a 12 h dark/light period-controlled room at 28˚C and 80% humidity. Blood feeding was performed using rabbit ears or artificially through glass feeders sealed with Parafilm and connected in a circulated water bath at 37˚C. Substitute of blood meal (SBM) is a previously described artificial diet with a chemically defined composition [47] and was used in experiments in which the presence of heme was modulated. For this study, only bovine albumin and gamma-globulin were used as protein sources, and no hemoglobin was added. Hemin was solubilized in 0.1 M NaOH and neutralized with 0.01 M sodium phosphate buffer (pH 7.4). Antibiotics (penicillin 200 U/ml and streptomycin 200 μg/mL) in autoclaved 5% sucrose solution were supplied for 3 days before feeding with blood or SBM.

Catalase activity

Midguts were dissected in cold 50% ethanol, and epithelia were separated from the blood bolus surrounded by the PM. The samples were immediately transferred to tubes with a protease inhibitor cocktail (50 μg/mL SBTI, 1 mM benzamidine, 1 mM PMSF), and pools of 7 tissues or PM were prepared. The midgut epithelial samples were directly homogenized, but the PM-enriched fraction samples were centrifuged 3x at 10000 × g for 5 min at 4˚C to remove as much of the blood bolus as possible. Hydrogen peroxide decomposition activity was measured from the rate of decrease in absorbance at 240 nm (e240 = 0.00394 liters $mmol^{-1}$ mm^{-1}).). Measurements were performed for 1 min in the presence of mosquito homogenates [48], and the protein concentration was determined according to Lowry [49]. For *in vitro* inhibition experiments, samples were incubated with different concentrations of 3-amino- 1,2,4-triazole for 30 min at 4˚C before enzymatic activity assays [20].

Double-strand RNA synthesis and injections

To synthesize dsRNA, a first PCR was performed using mosquito whole-body cDNA as a template. The product was diluted 100x and used in a second reaction with T7 primers. dsLacZ was used as a control and amplified from a cloned plasmid containing the LacZ gene. Double-strand RNA synthesis was performed using a MEGAscript T7 transcription kit (Ambion/Thermo Fisher Scientific, MA—USA). The reaction was performed overnight at 37˚C. Each product was precipitated with 1 volume of isopropanol and 1:10 volume of 3 M sodium acetate (pH 3). Four-day-old females were cold-anesthetized, and a double shot of 69 nl of 3 μg/μL dsRNA was injected into the mosquito thorax using a Nanoject II (Drummond Scientific, PA—USA). For double-silencing experiments, the dsRNA mixture containing both dsHPx1 and dsDUOX was lyophilized and then resuspended to half of the original volume. One day after injection, the females were blood-fed.

RNA extraction, cDNA synthesis, and qPCR

Total RNA was extracted from midgut samples using TRIzol reagent following the manufacturer's protocol. The RNA (1 μg) was treated for 30 min at 37˚C with DNAse, and cDNA was synthesized with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems/Thermo Fisher Scientific, MA—USA) using random primers. Quantitative PCR (qPCR) was performed with a StepOnePlus Real-Time PCR System (Applied Biosystems/Thermo Fisher

Scientific, MA—USA) using Power SYBR-green PCR master MIX (Applied Biosystems/ Thermo Fisher Scientific, MA—USA). The RP49 gene was used as an endogenous control, and the primers used in the qPCR analyses are listed in S1 Table.

HPx1 antiserum

Aedes aegypti HPx1 (AAEL006014) was cloned into pET15b at the NdeI (5') and BamHI (3') restriction sites. The export signal predicted by SignalP software [50] was removed from the purchased codon-optimized sequence (GenScript, NJ—USA). Plasmid pET15b containing the HPx1 protein-coding sequence was transformed into the *Escherichia coli* BL21(DE3) strain. Cells were grown at 37˚C in 2xYT medium containing 100 μg/L ampicillin. After reaching OD_{600} 0.4, the cells were cooled and supplemented with 0.5 mM isopropyl β-D-1-thiogalacto-pyranoside (IPTG), and the cells were incubated at 25˚C overnight. The cells were then harvested and resuspended in buffer A (200 mM Tris pH 8.0, 500 mM NaCl, 5 mM imidazole, 1% Triton X-100, 10% glycerol, 10 mM β-mercaptoethanol) supplemented with 2 mg/mL lysozyme. After breaking the cells by ultrasonic treatment, the insoluble fraction was collected by centrifugation. Because the recombinant HPx1 obtained by this protocol was not soluble, the corresponding protein band was cut from the SDS–PAGE gel, and the protein was extracted from the gel [51]. Immunization of BalB/C mice was performed by injecting two shots of 50 and 25 μg of antigen intraperitoneally, spaced by 21 days, using Freund's complete and incomplete adjuvant, respectively, in a 1:1 ratio (1 antigen:1 adjuvant). Two weeks after the second shot, blood was extracted by cardiac puncture, and the serum was isolated and frozen for further use.

HPx1 Western blotting

Pools of midgut epithelia and PMs were dissected and immediately placed in tubes containing a protease inhibitor cocktail. The samples were denatured at 95˚C in the presence of SDS sample buffer, and a volume equivalent to 1 midgut/slot was resolved by SDS–PAGE. The gel was blotted onto PVDF membranes (Bjerrum Schafer-Nielsen buffer—48 mM Tris, 39 mM glycine, 0.037% SDS—pH 9.2, 20% methanol) for 1 h at 100 V and blocked with 5% albumin in TBS-T (Tris 50 mM, pH 7.2, NaCl 150 mM, 0.1% Tween 20) overnight (ON) at 4˚C. The membranes were incubated with 1:5000 anti-HPx1 primary antiserum diluted in blocking solution for 5 h at room temperature. The primary antibody solution was removed, and the membrane was washed with TBS-T (3X) before incubation with alkaline phosphatase-conjugated anti-mouse secondary antibody (1:7500 in blocking solution) for 1 h at room temperature (RT). The membrane was washed and developed using NBT/BCIP alkaline phosphatase substrates.

Putative HPx1 gene promoter *in silico* analysis

The MEME motif-based algorithm [52] was used to analyze a 2500 bp sequence upstream of the 5' transcription start site of the HPx1 gene (*Aedes aegypti* genome, version AaegL3.4). The Fimo tool [53] was used to specifically search for cis-regulatory elements associated with ecdysone molecular signaling, as previously described [54].

PM permeability

HPx1-silenced mosquitoes were artificially fed rabbit blood containing 1 mg/ml dextran-FITC (Sigma, FD4), and the midguts were dissected 18 h after feeding. The midguts were fixed in 4% paraformaldehyde solution in 0.1 M cacodylate buffer for 2 h and placed ON in a 15% sucrose solution in phosphate-buffered saline (PBS; 10 mM Na-phosphate buffer, pH 7.2, 0.15

M NaCl) at RT. Then, they were incubated in a 30% sucrose solution for 30 h. The following day, the midguts were infiltrated with 50% Optimal Cutting Temperature/OCT (Tissue-TEK, Sakura Finetek, CA—USA) in 30% sucrose solution for 24 h and then ON in 100% OCT. The samples were frozen at -70˚C until use, and serial 14-μm-thick transverse sections were obtained using an MEV Floor Standing Cryostat (SLEE Medical, Nieder-Olm—Germany). The slices were placed on glass slides and mounted with Vectashield containing DAPI mounting medium (Vector laboratories, CA—USA). The sections were examined in a Zeiss Observer.Z1 with a Zeiss Axio Cam MrM Zeiss through excitation BP 546/12 nm, beam splitter FT 580 nm, and emission LP 590 nm.

Reactive oxygen species measurement

Midguts were dissected at 18 h after blood feeding and incubated with 50 μM dihydroethidium (hydroethidine; DHE; Invitrogen/Thermo Fisher Scientific, MA—USA) diluted in 5% fetal bovine serum-supplemented Leibovitz 15 medium for 20 min at room temperature in the dark. The dye medium was removed, and the midguts were gently washed in dye-free medium. The fluorescence of the oxidized DHE was acquired using a Zeiss Observer.Z1 with a Zeiss Axio Cam MrM Zeiss, and the data were analyzed using AxioVision software in a Zeiss-15 filter set (excitation BP 546/12 nm; beam splitter FT 580 nm; emission LP 590 nm) [7].

Mitosis labeling

HPx1-silenced mosquitoes were blood-fed, and the midguts were dissected 18 h after feeding. The midguts were fixed in 4% paraformaldehyde solution for 30 min, permeabilized with 0.1% Triton X-100 for 15 min at RT, and blocked ON at room temperature in a solution containing PBS, 0.1% Tween 20, 2.5% BSA, and 10% normal goat serum. All samples were incubated overnight with a mouse anti-PH3 primary antibody (1:500, Merck Millipore, Darmstadt—Germany) diluted in blocking solution at 4˚C and then washed 3x for 20 min each in washing solution (PBS, 0.1% Tween 20, 0.25% BSA). The midguts were incubated with a secondary goat anti-mouse antibody conjugated with Alexa Fluor 546 (Thermo Fisher Scientific, MA—USA) for 1 h at room temperature at a dilution of 1:2000, and nucleic acids were stained with DAPI (1 mg/ml, Sigma) diluted 1:1000. PH3-positive cells were visualized and counted using the Zeiss fluorescence microscope mentioned above [38].

Virus infection and titration

Zika virus (ZIKV; Gen Bank KX197192) was propagated in the *Aedes albopictus* C6/36 cell line for 7 days in Leibovitz-15 medium (Gibco #41300–039, Thermo Fisher Scientific, MA—USA; pH 7.4) supplemented with 5% fetal bovine serum, tryptose 2.9 g/L, 10 mL of 7.5% sodium bicarbonate/L; 10 mL of 2% L-glutamine/L, 1% nonessential amino acids (Gibco #11140050, Thermo Fisher Scientific, MA—USA) and 1% penicillin/streptomycin [20] at 30˚C. Dengue 2 virus (DENV, New Guinea C strain) was propagated in C6/36 in MEM media (GIBCO #11095080, Thermo Fisher Scientific, MA—USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin for 6 days [55]. The cell supernatants were collected, centrifuged at 2,500 × g for 5 min, and stored at -70˚C until use. Mosquitoes were infected with 10^5 PFU/ml ZIKV or $2x10^7$ PFU/ml DENV in a reconstituted blood meal prepared using 45% red blood cells, 45% of each virus supernatant, and 10% rabbit serum (preheated at 55˚C for 45 min). Four days after Zika infection or seven days after dengue infection, the midguts were dissected and stored at -70˚C in 1.5 ml polypropylene tubes containing glass beads and DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The samples were

thawed and homogenized, serially diluted in DMEM, and incubated in 24-well plates with a semiconfluent culture of Vero cells (for ZIKV samples) or BHK21 cells (for DENV) for 1 h at 37˚C and then incubated with DMEM 2% fetal bovine serum + 0.8% methylcellulose (Sigma, M0512) overlay for 4 days at 37˚C and 5% CO_2 in an incubator. The plates were fixed and stained for 45 min with 1% crystal violet in ethanol/acetone 1:1 (v:v).

Statistical analyses and experimental design

All experiments were performed in at least three biological replicates, and samples correspond to pools of 5–10 insects. The graphs and statistical analyses were performed using GraphPad 8 software. For qPCR experiments, relative gene expression was calculated by the Comparative Ct Method [56], and the result is expressed as the mean of ΔΔCt values compared to a housekeeping gene (RP-49, AAEL003396) [57].

Supporting information

S1 Fig. A) Catalase-specific activity comparison of the intestinal epithelia and PM at 24 h ABM. B) *In vitro* sensitivity of midgut epithelia and PM samples to aminotriazole, a catalase/peroxidase inhibitor. C) Mosquitos were treated with (or without) an antibiotic cocktail in a sugar meal, and PM catalase activity was assayed at 24 h ABM (BF n = 10; +AB n = 11). (D) Catalase activity comparison of the PM at 24 h ABM for mosquitoes fed blood or SBM, a chemically defined artificial diet (BF n = 21; +AB n = 17). E) HPx1 western blot of midgut epithelia and PM protein extracts, referring to Fig 1E. F) HPx1-silencing efficiency in the mosquito gut 24 h ABM.
(TIF)

S2 Fig. A) Schematic illustration of the E75 and ecdysone receptor-binding motifs location in the promoter region of HPx1. Numbers refer to nucleotide positions relative to the transcription start site. Table show output of the FIMO analysis including p-values and sequences for each putative TF binding site. B) E75 silencing efficiency in midguts at 24 h ABM. C) HPx1 expression in midguts at 24 h ABM. Control mosquitoes were fed a regular sucrose solution before blood feeding. Ab mosquitoes were pretreated with antibiotics before blood feeding. Ab + Bac mosquitoes were pretreated with antibiotics and fed blood containing *Enterobacter cloacae at* 1 OD/ml (Ctr n = 6; Ab n = 6; Ab+Bac n = 5). Data are the mean +/- SEM.
(TIF)

S3 Fig. A) Dityrosine immune staining of mosquito midgut epithelia 18 h ABM. The protocol used was the same published by Kumar et al., 2010 [22] for IMPer characterization in *Anopheles gambiae*. B) Western blotting detection of dityrosine in protein extracts of PM pools of dsLacZ and dsHPx1. Briefly, 25μg of protein of each sample was resolved in an SDS gel and wet transferred to a PVDF membrane for 1h, 100V in cold room. The primary antibody was diluted in blocking solution (1:1000) ON at 4˚C. The membrane was developed using HRP-SuperSignal West Dura Substrate (Invitrogen). C) Peritrophic matrix staining by WGA--FITC. Midguts were dissected 18 h ABM, fixed ON in 10% neutral buffered formalin solution at room temperature, paraffin-embedded, and sectioned. The WGA staining was performed as in Talyuli et al., 2015 [47].
(TIF)

S4 Fig. Viral infection in Chitin Synthase-silenced insects. A) Dengue 2 titers in midguts 7 days postinfection. Viral titers were assessed by the plaque assay. Each dot represents an individual mosquito gut, and bars indicate the median. * $p < 0.05$; Mann–Whitney test. The

prevalence statistical analysis was performed by the Chi-square test followed by Fisher's exact test.
(TIF)

S1 Table. Primer list.
(TIF)

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