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Elucidating The Role of a Nematode Secreted PLA_2 and Lipid Signaling in Immunomodulation of *Drosophila melanogaster*

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DEDICATION

I'd like to dedicate my dissertation work to my mentor have guided me throughout this scientific journey and process, Dr. Adler Dillman**.** I believe you were sent in my life by God to help get me to where I am today. To me there is no other explanation how I came to your lab at the time when I most needed to, and how I was able to accomplish scientifically what I always envisioned at the pace I did if not for it being God's plan.

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ABSTRACT OF THE DISSERTATION

Elucidating The Role of a Nematode Secreted PLA_2 and Lipid Signaling in Immunomodulation of *Drosophila melanogaster*

by

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Doctor of Philosophy, Graduate Program in Biomedical Sciences
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Dr. Adler Dillman, Chairperson

Parasitic nematodes are a global health concern and can infect a variety of organisms such as insects and mammals. As a result of infection, they can cause significant morbidity and mortality. Upon successful infection they can release excreted/ secreted proteins (ESPs) into the host, which enables them to evade or suppress host immunity and cause toxicity. Despite some characterization of ESPs in certain parasitic nematode species, very little is known about the mechanisms behind these interactions. Parasitic nematodes that infect insects called entomopathogenic nematodes (EPNs), have been employed to better understand molecular mechanism. Research with EPNs utilize insect model systems which enables circumvention of logistic and technical challenges encountered with using mammals. EPNs also have high homology with vertebrate parasitic nematodes which leads to the application of translational research. This work focuses on an ESP released by the EPN *Steinernema carpocapsae*, that displayed immunomodulatory effects in the model host *Drosophila melanogaster*. The ESP is a secreted phospholipase A_2 ($sPLA_2$) enzyme that I named Sc-$sPLA_2$ and displayed immunosuppressive effects by reducing the number of hemocytes in the host, and likely

by increasing circulation of an anti-inflammatory lipid. The $sPLA_2$ operates enzymatically by cleaving fatty acids directly from the membrane which resulted in immunosuppressive effects on the humoral and cellular response. Analysis of fly hemolymph post injection of Sc-$sPLA_2$ showed increase of eicosanoid and oxylipin precursors, and the increase of an anti-inflammatory fatty acid. In addition, the analysis of fly hemolymph post infection revealed several lipids that are depleted with the ability to rescue immunity upon treatment. This work also attempted to characterize endogenous lipid signaling mechanisms by identifying lipids, prostaglandins and endogenous $sPLA_2$ enzymes that significantly improve the outcome of infection. Thus far this work has been able to establish that an eicosanoid precursor was able to stimulate the cellular response, furthering our understanding of how lipid signaling is immunomodulatory in *D. melanogaster*.

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Introduction

Review of the Role of Parasitic Nematode Excretory/Secretory Proteins in Host Immunomodulation

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This Introduction section is a literature review authored by me and published in the Journal of Parasitology on March 2022. This review is important as it validates the reasoning of using the insect model system as a translational research approach for my research work in the subsequent chapters. It also validates the reasoning for using insect parasitic nematodes to circumvent technical challenges for characterizing molecular mechanisms of excretory/secretory proteins.

ABSTRACT

Parasitic nematodes infect a variety of organisms including insects and vertebrates. To survive, they evade host immune responses to cause morbidity and mortality. Despite the vast clinical knowledge regarding nematode infections and their biological makeup, molecular understanding of the interactions between host and parasite remains poorly understood. The utilization of model systems has thus been employed to help elucidate the molecular interactions of the host immune response during parasitic nematode infection. Using model systems, it has been well established that parasitic nematodes evade host immunity by releasing excretory/secretory proteins (ESPs), which are involved in immunomodulation. Model systems have enabled researchers to characterize further the underlying mechanisms ESPs use to facilitate evasion and modulation of the host immune response. This review assessed notable ESPs from parasitic nematodes that infect vertebrates or insects and have been studied in mechanistic detail. Being able to characterize how ESPs affect the immune systems of hosts on a molecular level increases our understanding of host–parasite interactions and could lead to the identification of novel therapeutic targets and important molecular pathways.

SIGNIFICANCE

Infections caused by parasitic nematodes are a widespread global health concern that continues to afflict humans. It has been estimated that parasitic nematodes infect more than 25% of the global population, with the concentration of infections being primarily in the global south [1-3]. The difficulty of detecting parasitic infection during the early

stages compounds the health effects. Part of what makes nematode infections difficult to diagnose early on is the ability of parasitic nematodes to evade the host immune system, allowing them to go undetected, which in turn leads to physiological complications that cause morbidity and mortality [4]. The global health ramifications of parasitic nematodes are further exacerbated by the possibilities of recurrent reinfection and emerging drug-resistant infections. Nematodes are thus very troublesome parasites with the ability to compromise the immune systems of insects and vertebrates [5-7].

The difficulty in identifying the underlying molecular mechanisms is in part due to the lack of good model systems with established genetic, genomic, and proteomic tools that overcome logistic obstacles such as cost and time [8, 9]. These obstacles have been notably dealt with in other areas of investigation such as behavioral ecology and neurobiology, where insect-parasitic nematodes that are closely related to vertebrate-parasitic nematodes are used as models [10-13]. As a result, plausible methods for the characterization of host immunomodulation by nematodes have been made more efficient. One potential use of these model systems is to study the impact of excretory/secretory proteins (ESPs) on a host in the context of infection.

Individual components of ESPs have been observed to have mechanisms of modulating the immune system of infected hosts [14]. The immunomodulation promotes the survival of the parasitic nematode by strategically altering the activation of host immune responses upon infection [14]. ESPs have a broad spectrum of effects and have the ability to impact host responses in a multifaceted context, including the response to concurrent diseases by bystander pathogens [15]. This is highlighted by ESP-driven anti-

inflammatory responses being implicated in the low occurrence of inflammatory bowel disease in populations with high rates of nematode infection [16]. Overall, specific ESPs involved in nematode immunomodulation generally vary with regards to the mode of action and type of host [17-19]. ESPs released also vary between the various life stages of the parasitic nematode, and even between sexes [20, 21]. The need for stage-specific ESPs most likely aligns with the specific outcomes required for the life stage [20, 21]. Overall, few of these proteins have been studied in mechanistic detail. This review examined select immunomodulatory ESPs from nematode parasites of insects and vertebrates that have been mechanistically characterized and assessed the potential of insect-parasitic nematodes to serve as model systems for molecular characterization of immunomodulatory ESPs [22].

MOLECULAR INTERACTIONS OF ESPS IN INSECTS

A specialized subset of insect-parasitic nematodes called entomopathogenic nematodes (EPNs) are characterized by their ability to kill hosts quickly, and their utilization of symbiotic bacteria to facilitate their parasitic lifestyles [23, 24]. Most EPNs enter an insect through natural openings, and once inside they release highly pathogenic bacteria along with ESPs into the insect's hemolymph. Although it was originally thought that the bacterial symbionts were the primary source of toxicity to insect hosts, with the nematodes serving primarily as vectors, recent studies showed that ESPs of EPNs are highly toxic to insects [25, 26]. Infection by EPNs does not go unnoticed by insects, however, as the insect's innate immune system uses a series of mechanisms that detect the

nematode and bacterial partners to restrain their dissemination [27, 28]. These immune response mechanisms fall under 2 categories: humoral and cellular [29, 30]. The humoral immune response activates genes needed for synthesizing and secreting antimicrobial peptides (AMPs) from the fat body into the hemolymph [31-33]. Cellular immune responses are regulated by hemocyte function [34]. Hemocytes are the main component of the cellular response, and they are implicated in several functions like cell aggregate formation, phagocytosis, melanization, and encapsulation to help fight off infections [35, 36]. Melanization occurs after the production of phenol-oxidase (PO) that is produced by the cleavage of the proenzyme prophenoloxidase [35], which is a key component of the insect immune system [37, 38]. Prophenoloxidase catalyzes melanization by mediating the oxidation of mono- and diphenols to quinones; they then polymerize to form melanin-generating reactive oxygen species (ROS) [39]. In *Drosophila* hemolymph coagulation, after the initial phase where cross-linking depends on transglutaminase activity, PO activity becomes the key component in the subsequent phase for further cross-linking, hardening, and melanization of the clot matrix [40]. Hemolymph coagulation is important in the insect immune response as it stops bleeding, seals wounds, and prevents the dissemination of pathogens and entry of microbial invaders at the wound site [40]. To have a successful infection, EPNs must evade, suppress, or modulate the insect immune response, at least temporarily to survive, release their mutualistic bacteria, and complete their life cycle. These bacteria are located in a receptacle near the pharyngeal bulb and are necessary for the growth and development of the nematode during infection [41]. Thus, EPNs are obliged to deploy rapid immunomodulatory strategies to protect the small

cohort of symbiotic bacterial cells they release into the host, depressing host immunity, at least temporarily, so that the bacteria can resume growth and deploy their immunomodulatory arsenal to aid in protection from host immunity.

It is important to note that EPNs release ESPs during the infective juvenile stage (IJ) [25, 26]. As IJs, EPNs are in arrested development until a host is found; upon entering the host the IJs become activated for release of ESPs [25, 26]. The subsequent proteins that will be described were discovered in the ESPs released by specific EPNs. Each protein displayed notable immunomodulatory properties during experimental studies that will be briefly discussed (Table I).

Table I EPN ESP characterization

Table highlights entomopathogenic nematodes and excretory/secretory proteins (ESPs). Molecular immunomodulatory effects are what is observed for the corresponding type of ESP, and thus based on the type of the pathways and molecular/cellular mechanisms they affect.

Infected host	Parasitic ESPs released	Molecular immunomodulatory effects*	References
Insect	Sc-CHYM	Decrease of hemocyte encapsulation, PPO inhibition.	Balasubramanian et al. (2009)
	Sc-SRP-6	No melanin deposition, disruption of clotting.	Toubarro et al. (2013a)
	Sc-KU-4	Inhibition of hemocyte aggregation and encapsulation.	Toubarro et al. (2013b)
	Trypsin-like serine protease	PPO inhibition, change in hemocyte morphology, reduced hemocyte spreading and recognition.	Balasubramanian et al. (2010)
	Hb-ilys-1	Suppression of PO activity.	Kenney et al. (2021)
	Hb-sc-1	Suppression of PO activity, reduced AMP upregulation, reduced phagocytic activity.	Kenney et al. (2021)
	Hb-ugt-1	Suppression of *Br-c*, reduced AMP upregulation.	Kenney et al. (2020)

*Abbreviations: PPO: prophenoloxidase, PO: phenoloxidase. AMP: antimicrobial peptides.

Trypsin-like serine protease and Sc-CHYM

Research to identify immunomodulatory proteins led to the discovery of a trypsin-like serine protease secreted by *Steinernema carpocapsae* IJs, during infection of *Galleria*

mellonella larvae [42]. Endogenous serine proteases and serine protease inhibitors are important in immune response, as they activate proenzyme prophenoloxidase [35], converting it to phenoloxidase (PO), and this activation results in hemocyte encapsulation, and melanization [43]. Although many trypsin serine proteases that have been discovered have been assigned a group or family, the trypsin-like serine protease that was described in Balasubramanian et al. (2010) has not been fully characterized for classification. The trypsin-like serine protease displayed the ability to suppress 38.9–52.6% of proPO experimentally, leading to interruption of the process of melanization and ultimately reduced EPN encapsulation by hemocytes [42]. It altered the morphology of *G. mellonella* hemocyte F-actin filaments from a highly organized state to a disorganized state and caused a change of hemocyte spindle shape, which coincided with inhibition in the hemocyte spreading [42]. This also resulted in reduced recognition of the EPN *Heterorhabditis bacteriophora* by *G. mellonella* hemocytes by 55% [42]. The trypsin-like serine protease thus significantly impairs host immunity by decreasing hemocyte spreading, encapsulation, and recognition of EPNs during infection.

Another study of ESPs secreted by *S. carpocapsae* reported a discovery of a chymotrypsin serine protease virulence factor named Sc-CHYM [14, 44]. Because this protein is a serine protease such as the 1 described prior, it is likely they both affect the activation of the proPO-PO cascade by competing with the endogenous serine proteases. In vitro, Sc-CHYM displayed the capability to inhibit proPO by suppressing its enzymatic activity [44]. In vivo, Sc-CHYM reduced the melanization and encapsulation of protease-treated beads that were injected into *G. mellonella*; normally such foreign

objects are encapsulated and melanized [44]. Sc-CHYM was thus shown to weaken the cellular immune response of the insect host and increase the success of parasitism by *S. carpocapsae*.

Sc-SRP-6

Another ESP from *S. carpocapsae*, Sc-SRP-6, was shown to have 2 roles in protecting EPNs from host immunity. The first role is the inhibition of hydrolysis of food particles by reducing the activity of insect digestive enzymes [45]. Protection from digestive enzymes keeps IJs and their ESPs safe from metabolic breakdown when they enter the alimentary canal of the host. The second role of Sc-SRP-6 is interfering with clot formation in infected insects by binding with hemolymph plasma proteins, forming complexes that prevent the incorporation of melanin into the clot matrix, which is essential for encapsulation and nodulation immune processes [36, 45-47]. Inhibiting clot formation weakens host cellular immunity, adding further protection to IJs during parasitism via host immunomodulation.

Sc-KU-4

Another protein of interest, which belongs to the Kunitz-type serine family of protease inhibitors, is Sc-KU-4. This protease inhibitor is most highly expressed by the invasive stage, the IJ, of *S. carpocapsae*. Sc-KU-4 was reported to inhibit hemocyte aggregation in *G. mellonella* hemolymph [48]. Beads treated with Sc-KU-4 remained individualized in *G. mellonella* plasma, whereas nontreated beads were aggregated and entrapped by

clotting material, suggesting Sc-KU-4 protects foreign bodies from host clotting mechanisms [48]. Lastly, Sc-KU-4-treated beads were pulled down from insect plasma and observed to be strongly bound to 2 proteins linked to immune recognition: A homolog of a masquerade-like protein (MSPH) and a homolog of a serine protease-like 1b (SPH-1) [48]. These findings suggest that Sc-KU-4 targets insect immune recognition proteins in the plasma such as antimicrobial peptides (AMPs), inhibits hemocyte aggregation, and prevents encapsulation of EPNs. Protecting IJs from recognition proteins enables them to hide from the host, thus preventing an adequate immune response to parasitic infection.

Hb-sc-1 and Hb-ilys-1

The EPN *Heterorhabditis bacteriophora* has also been utilized for the discovery of novel immunomodulatory ESPs by transcriptomic analysis. Transcriptome studies were able to identify multiple secreted protein factors that were upregulated during parasitism [49]. Two notable proteins that were recently characterized are a putative lysozyme (Hb-ilys-1) and serine carboxypeptidase (Hb-sc-1) [50]. The potential immunomodulatory capabilities of these proteins were assessed utilizing *Photorhabdus luminescens* infection of *Drosophila melanogaster*. Both recombinant proteins caused increased mortality during in vivo co-injections of *D. melanogaster* with *P. luminescens*, when compared to injections of *D. melanogaster* with *P. luminescens* alone [50]. Both Hb-ilys-1 and Hb-sc-1 suppressed PO activity, which correlated with a reduced melanization response during infection. In addition to reduced PO activity, Hb-sc-1 also reduced the upregulation of

certain AMPs (*Diptericin*, *Attacin*, and *Drosomycin*), indicating inadequate activation of the immune response [50]. It was also found that Hb-sc-1 reduced phagocytic activity so that hemocytes were less effective at phagocytosing pHrodo-labeled *Escherichia coli*. This indicates that Hb-sc-1 might be broadly interfering with the cellular response of the fly during infection [50]. Further molecular experimentation is needed to understand how PO activity is suppressed by both enzymes, as well as to elucidate how Hb-sc-1 causes reduced upregulation of AMPs and reduced phagocytic activity. Both Hbsc-1 and Hb-ilys-1 cause measurable effects on host immunity during infection resulting in reduced survival.

Hb-ugt-1

Another protein released by *H. bacteriophora* that displayed immunomodulatory effects is a putative UDP-glycosyltransferase called Hb-ugt-1. A recent study showed that injection of *D. melanogaster* with recombinant Hb-ugt-1 resulted in reduced upregulation of the AMPs Diptericin, Attacin, and Metchnikowin [51]. To assess the physiological effects of this, *D. melanogaster Relish* mutants lacking an immune deficiency (Imd) – based response, were injected with recombinant Hb-ugt-1 to assess survival. The survival of these injected flies was significantly lower over 6 days in comparison to regular survival for wild-type flies, though the reason for reduced survival in this mutant context is not fully understood [51]. In addition to AMP suppression, *D. melanogaster* larvae injected with recombinant Hb-ugt-1 showed suppression of the ecdysone-transcription factor *Broad-Complex* (*Br-c*), which upregulates components of the immune response

including the Peptidoglycan Recognition Protein LC (PGRP-LC) and some AMPs [51]. Thus, the suppression of Br-c by Hb-igt-1 may be responsible for the reduction of AMP upregulation. Through the reduction of AMP upregulation, Hb-ugt-1 is likely able to compromise the host immunity during infection.

MOLECULAR INTERACTIONS OF EXCRETORY/ SECRETORY PROTEINS IN VERTEBRATES

With over 1 billion people infected worldwide, vertebrate-parasitic nematodes continue to be a major public health concern globally, specifically in nations in the global south [1-3]. Understanding how these nematodes evade vertebrate immunity is thus a major priority. The invasion of host tissues by parasitic nematodes activates the complement system, which identifies pathogens and directs the innate immune response. Leukocytes (encoded by the MHC class I and II genes in humans) are then recruited to the site of infection to release cytokines to enhance an inflammatory response along with a variety of other processes [52]. Participation of mast cells and eosinophils also occurs because of their roles as potent effectors of a range of cytokines and chemokines. Direct activation of leukocytes is triggered by host tissue damage by the invading nematode, which then leads to the recruitment of other kinds of leukocytes, such as neutrophils, macrophages, basophils, innate lymphoid cells, and dendritic cells, which leads to the production of toxic free radicals, phagocytosis, and the eventual development of adaptive immune response by the production of antibodies [53-55]. Vertebrate-parasitic nematodes,

however, have evolved immunomodulatory mechanisms, effected through their ESPs (Table II), that can interrupt 1 or more effectors of the innate immune response [56].

Table II Vertebrate nematode ESP characterization

Table highlights notable vertebrate parasitic nematode excretory/secretory proteins (ESPs). Molecular immunomodulatory effects are what is observed for the corresponding type of ESP, and thus based on the type of the pathways and molecular/cellular mechanisms they affect.

Infected host	Parasitic ESPs released	Molecular immunomodulatory effects	References
Vertebrates	ES-62	Inhibition of B and T cell activation and proliferation. Inhibition of mast cell degranulation and the release of pro-inflammatory mediators. Inhibition of IL-12p70 and pro-inflammatory Th1 responses. ES-62 regulates gene induction by modulating the binding of NF-B to the IL-12 promoter.	Goodridge et al. (2005a) Goodridge et al. (2005b) Harnett et al. (2004) Marshall et al. (2005) Melendez et al. (2007) Whelan et al. (2000) Wilson et al. (2003a) Wilson et al. (2003b)
	Cystatins	Reduced T cell priming. Inhibition of T cell proliferation. Enhanced production of anti-inflammatory Il-10. Reduced induction of active immune response.	Dainichi et al. (2001) Manoury et al. (2001) Schnoeller et al. (2008) Schönemeyer et al. (2001)
	Ac-AIP-1	Reduction of local infiltration of inflammatory cells. Suppression of pro-inflammatory cytokines. Production of anti-inflammatory IL-10.	Ferreira et al. (2017)
	Ac-AIP-2	Suppression of T cell proliferation. Reduced DC co-stimulatory marker expression.	Navarro et al. (2016)

ES-62

The glycoprotein ES-62 is an ESP released by the postinfective life-cycle stages of the rodent filarial nematode *Acanthocheilonema viteae* with immunomodulatory properties highlighted by the ability to interact with a variety of immune cells, thus being able to regulate the host immune system via the cellular response [57, 58]. ES-62 specifically alters molecular events that control B cell and T cell receptor signaling, which leads to significant inhibition of B cell and T cell activation and proliferation [59]. ES-62-mediated modulation requires the presence of Toll-like Receptor TLR4, but not TLR2 and TLR6, and can affect antigen-presenting cells, as well as the inhibition of mast cell

degranulation by the formation of a complex with TLR4 at the plasma membrane [60, 61].

ES-62 is heavily conjugated with and modifies phosphorylcholine (PC), which leads to inhibition of the proliferation of $CD4^+$ T cells and conventional B2 cells in vivo. It also reduces IL-4 of $CD4^+$ cells and interferon-gamma (IFN-γ) production [62-65]. ES-62 also promotes the proliferation of peritoneal B1 cells and their subsequent production of IL-10 [65]. ES-62 also targets antigen-presenting cells (APCs) to inhibit their ability to produce IL-12p70 in response to lipopolysaccharides [66]. This is done with pretreatment of DCs and macrophages with ES-62, where ES-62-pulsed bone marrow–derived DCs can drive Th2 differentiation in vitro [67, 68]. Utilizing its PC residues, ES-62 interacts with toll-like receptor (TLR) 4 to inhibit pro-inflammatory Th1 responses. In mast cells, binding of TLR4 by ES-62 results in degradation and sequestration of intracellular protein kinase C-α (PKCα), which as a result inhibits degranulation and release of inflammatory mediators [60, 61].

Although ES-62 can impair the host immune response, it has also shown the ability to reduce the outbreak of various autoimmune or allergy-related diseases [69]. ESPs such as ES-62 thus not only have a role in host immunomodulation, but potentially can be utilized to design novel anti-inflammatory drugs [59, 70]. Ultimately, ES-62 displays the ability to regulate B cell and T cell receptor signaling, as well B cell and T cell activation and proliferation, significantly.

Cystatins

Cystatins are cysteine protease inhibitors. Cystatins have been found among the ESPs of third-stage larvae (L3) vertebrate-parasitic nematodes and have been identified to have immunomodulatory properties on the cellular response [71, 72]. They inhibit 2 classes of cysteine proteases: Legumains, which are utilized for antigen processing and presentation, and cathepsins L and S, which are utilized for processing polypeptides. Inhibition of legumains reduces the formation of the MHC class II molecules, which reduces the induction of an active immune response [73]. Cystatins can also enhance the production of anti-inflammatory cytokine IL-10, which in turn restricts T cell–mediated responses [74]. Cystatins secreted by *Heligmosomoides polygyrus* have been shown to modulate the activity of dendritic cells. Recombinant cystatin exposed to dendritic cells resulted in the expression of fewer MHC class II molecules as well as CD 40 and CD 86, 2 proteins necessary for T cell differentiation [75, 76]. Another cystatin secreted by *A. vitae* alters the expression of key cytokines resulting in modulation of pro-inflammatory effects [77]. Recombinant cystatin resulted in downregulation of the pro-inflammatory cytokines iNOS and cycolooxygenase synthase (COX)-2 and induced an upregulation of IL-10, which further promoted an anti-inflammatory effect in microglia [14].

Cystatins produce immunomodulatory effects through 2 mechanisms [18, 78]. First is the inhibition of cysteine proteases (cathepsins and aspartyl endopeptidase) necessary for host APC antigen processing and presentation, which results in reduced T cell priming [79, 80]. The second mechanism is the induction of immunosuppressive IL-10, reducing co-stimulatory molecule expression by APCS, and inhibiting T cell proliferation [81].

Immunomodulation in vivo has also been characterized by inhibition of both allergic lung inflammation and colitis, which is both mediated by Tregs and IL-10–producing macrophages [82]. Through their inhibition of cysteine proteases, cystatins can modulate T cell differentiation and proliferation.

Anti-inflammatory proteins (Ac-AIP-1 and Ac-AIP-2)

Gastrointestinal hookworms have evolved to cause minimal harm to their host in low-burden infections, through the secretion of immunomodulatory ESPs [83]. This allows for the long-term survival of the parasites in a host while potentially protecting the host from inflammatory diseases [83, 84]. Two anti-inflammatory ESPs Ac-AIP-1 and Ac-AIP-2 were found in the blood-feeding stage (L4) of hookworm *Acylostoma caninum*. They were recombinantly expressed and experimentally shown to display immunomodulatory effects on the cellular response [72, 85]. Recombinant Ac-AIP-1 was assessed in mouse models of colitis [83, 86]. Colitis inflammation was suppressed in these models by Ac-AIP-1 at 1 mg kg^{-1}, and local infiltration of inflammatory cells was significantly reduced. Colitic inflammation was assessed as weight loss, colon atrophy, edema, ulceration, and necrosis, as well as abdominal adhesion. Recombinant Ac-AIP-1 promoted the production of anti-inflammatory colon IL-10, transforming growth factor (TGF)-β, and thymic stromal lymphopoietin (TSLP). It also suppressed several cytokines, including the tumor necrosis factor (TNF)-α, IL-13, and IL-17A, granulocyte macrophage colony-stimulating factor (GM-CSF), CX motif chemokine (CXCL)-11, COX-2 mRNA transcripts, and IFN-γ. Ac-AIP-1 thus displayed immunosuppressing characteristics by

promoting the production of anti-inflammatory mediators IL-10 and TGF-β, and suppression of pro-inflammatory cytokines.

Ac-AIP-2 is 1 of the most abundant proteins in the *A. caninum* secretome (secreted proteome) and demonstrated immunomodulatory capabilities in a mouse model of asthma [84, 86]. Ac-AIP-2 suppressed airway inflammation, reduced DC co-stimulatory marker expression, and demonstrated ex vivo suppression of human T cell proliferation with dust mite allergy [84]. Mouse models showed that Ac-AIP-2 was primarily captured by mesenteric CD103+ DCs, that airway inflammation suppression was primarily dependent on DCs, and mesenteric lymph node originated (MLNs) Foxp3+ regulatory T cells [84]. Thus, potential anti-inflammatory therapeutic effects of Ac-AIP-2 were mechanistically characterized to be dependent on capture by mesenteric DC and Treg cells, which is also a mechanism by which Ac-AIP-2 modulates the immune response of the host upon secretion.

CONCLUSION AND FUTURE DIRECTIONS

The characterization of tens of immunomodulatory ESPs (Fig. 1) from among the hundreds that have been identified highlights the challenge of ESP mechanism elucidation. Major obstacles to research success include the cost and time required to characterize ESPs of vertebrate parasitic nematodes at the molecular level fully. Recent studies show the promise of insect model systems to rationally identify immunomodulatory ESPs for recombinant expression and characterization [25, 26, 87]. A process of in vitro activation of EPNs has been optimized, allowing for the time-friendly acquisition of high quantities of ESPs for downstream applications such as mass

spectrometry identification of protein composition or fractionation of ESPs for targeted identification [25, 26]. Although other methods of identification and collection are utilized with vertebrate systems, the insect system, along with proteomics and transcriptomics, presents a time- and cost-effective model for researchers to screen, identify, or isolate novel proteins [21, 25, 88-90]. Also, in vitro ESP collection methods for vertebrate-parasitic nematodes have yet to be experimentally validated regarding their relevance to in vivo conditions, where EPN insect model systems have been so validated [20, 21, 25, 26, 91]. EPN model systems have many advantages; there are still, however, areas of research where they can be developed. Immune priming, which is characterized by the increase in survival and host immune response after a second specific encounter, is a phenomenon in invertebrates that EPNs have recently been used to examine [92-94]. A recent study showed that an EPN did not generate immune priming, future experiments can elucidate factors for this or if they can elicit immune priming under certain conditions [94]. More research is needed regarding immune priming, but EPNs are still a promising and relevant model system that is closely related to nematode parasites of humans, and even releasing many of the same ESPs into their hosts [25, 95].

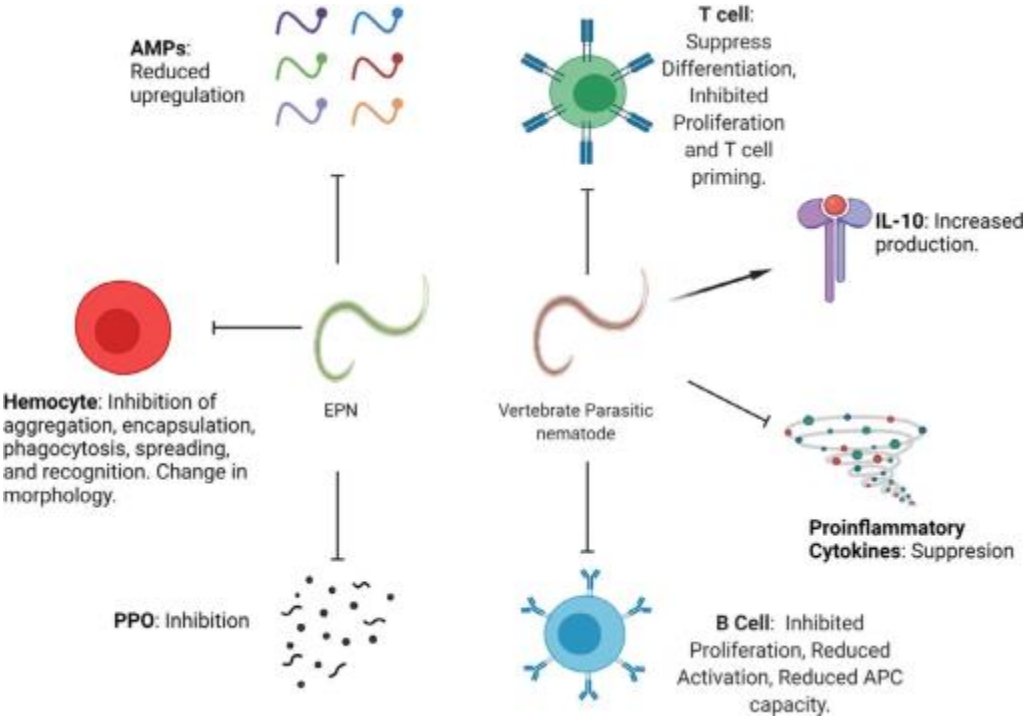

Figure 1 ESP effect on insects and vertebrates. Visual representation of the molecular effects on host immunity by excretory/secretory proteins (ESPs). Left side displays the molecular interactions of ESPs released by entomopathogenic nematodes (EPNs) on insect immunity. Right side displays the molecular interactions of ESPs released by vertebrate parasitic nematodes on mammalian immunity.

If successfully utilized, EPN–insect model systems can allow for the identification of novel mechanisms of immunomodulation and facilitate their characterization. With a better foundation for selecting individual proteins or molecules for experimentation, vertebrate studies can be more precise and efficient in elucidating molecular pathways involved with vertebrate-parasitic nematodes. This may allow for the development of vaccines that can promote parasitic nematode clearance, better treatments of infection, or better treatments of autoimmune disease using drugs derived from immunosuppressing ESPs.

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Chapter 1

Parasitic nematode secreted phospholipase A_2 suppresses cellular and humoral immunity by targeting hemocytes in *Drosophila melanogaster*

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This chapter contains work published in Frontiers in Immunology. I contributed to this publication as co-first author. The author contributions section contains an overview of contributions to the work, but I will outline in some more detail my contributions to the overall work. I aided Sophia's contributions (SCP) by counting flies that were injected for survival and toxicity assays. I aided in plating and counting for the CFU colony experiments. I aided the PO assay by recording the absorbance of plate assay. I performed the RNA extraction and RT-qPCR for the AMP immunoassay and did the data analysis. I aided in the pHrodo phagocytosis assay by helping visualize and taking photos of the fly. I also processed the data with ImageJ and analyzed the data for graphing. I designed and executed all protein recombinant expressions and purification. I designed

and executed all enzymatic assays both for the enzchek activity kit and mass spectrometry lipidomics assays and performed data analysis on them. I designed and executed the cell lysis assay with the *Drosophila melanogaster* S2 cells, and processed and analyzed the data. For the hemocyte count with Pakeeza (PA) I aided by performing the trypan blue live count cell procedure with the hemocytes, and processed and analyzed the data.

ABSTRACT

A key aspect of parasitic nematode infection is the nematodes' ability to evade and/or suppress host immunity. This immunomodulatory ability is likely driven by the release of hundreds of excretory/secretory proteins (ESPs) during infection. While ESPs have been shown to display immunosuppressive effects on various hosts, our understanding of the molecular interactions between individual proteins released and host immunity requires further study. We have recently identified a secreted phospholipase A2 ($sPLA_2$) released from the entomopathogenic nematode (EPN) *Steinernema carpocapsae* we have named Sc-$sPLA_2$. We report that Sc-$sPLA_2$ increased mortality of *Drosophila melanogaster* infected with *Streptococcus pneumoniae* and promoted increased bacterial growth. Furthermore, our data showed that Sc-$sPLA_2$ was able to downregulate both Toll and Imd pathway-associated antimicrobial peptides (AMPs) including drosomycin and defensin, in addition to suppressing phagocytosis in the hemolymph. Sc-$sPLA_2$ was also found to be toxic to *D. melanogaster* with the severity being both dose- and time-dependent. Collectively, our data highlighted that Sc-$sPLA_2$ possessed both toxic and immunosuppressive capabilities.

INTRODUCTION

Nematode parasitism is an important global health and agricultural issue, responsible for significant morbidity and mortality to humans, illness to livestock, and a reduction of global crop yields [1–3]. Parasitic nematodes have ravaged human populations, with over 1.5 billion people being infected by soil-transmitted helminths alone [4]. This issue is

further compounded by recurrent reinfection and emerging drug resistance. Parasitic nematodes are thus very effective parasites, capable of evading and compromising the immune response of various hosts including insects and vertebrates [5–7]. Despite the vast clinical knowledge on parasitic nematode infections, our understanding of the mechanisms that underlie helminths' ability to modulate host immunity remains incomplete. By elucidating the molecular mechanisms of parasitic nematode immunomodulation, more effective anti-helminthic therapeutics can be produced, as well as potential therapeutics for treating human immune pathologies such as autoimmune diseases.

Parasitic nematodes are able to evade and alter host immunity *via* their release of excretory/secretory proteins (ESPs). ESPs consist of a variety of proteins that have effects ranging from metabolic breakdown of host tissue to immunomodulatory capabilities. Immunomodulatory proteins are able to promote the survival of parasitic nematodes during infection by strategically altering the activation of the host immune response [8]. Characterization of individual proteins has remained challenging due to the technical obstacles of vertebrate model systems for testing hypotheses of potential effector proteins. Utilization of insect model systems however, has resulted in molecular characterization of individual proteins found in EPN ESPs [9]. Due to the high homology EPN ESPs have with nematode parasites of vertebrates such as *Strongyloides stercoralis*, the molecular mechanisms of their ESPs are likely conserved [10–12]. Effector proteins of the EPN *Steinernema carpocapsae* were assessed using the host *Drosophila melanogaster* due to its highly conserved innate immune system, with key immune

signaling pathways and transcription factors resembling those in mammals [13]. The *D. melanogaster* immune response includes a humoral and a cellular component [14, 15]. The humoral immune response activates genes needed to synthesize and secrete antimicrobial peptides (AMPs) from the fat body into the hemolymph [16–18]. Cellular immune responses are regulated by hemocyte function [19]. Hemocytes regulate several cellular response mechanisms including cell aggregate formation, phagocytosis, melanization, and encapsulation which aid in fighting infections [20, 21]. Melanization occurs after the production of phenoloxidase (PO) *via* up-regulation of prophenoloxidase [22, 23]. PO serves as a catalyst for melanization by mediating the oxidation of mono- and di-phenols to quinones and is then followed by subsequent polymerization to form antimicrobial melanin [24, 25]. This process ultimately results in the generation of reactive oxygen species (ROS) lethal to microbes [24]. Activation of the immune response is generally regulated by two NF-kB signaling pathways: Toll and Imd which are similar to human toll-like receptors (TLR) and tumor necrosis factor (TNF) signaling respectively [14]. Activation of these pathways is thought to be pathogen specific and depend on external cellular properties such as cell wall composition. Systemic production of specific AMPs *via* the humoral response is dependent on whether the Toll or Imd pathway is activated [26–28]. EPNs must evade, suppress, and/or modulate the insect immune response by releasing effector proteins during infection to survive and complete their life cycle.

One family of effector proteins identified in the EPN *S. carpocapsae* was the secretory phospholipase A2 proteins ($sPLA_2$) [29]. The $sPLA_2$ proteins are low molecular weight

(13-19 kDa), and are Ca2+-dependent secretory enzymes that consist of 12 groups [30]. In insects, PLA_2 function has been shown to play a role in digestive physiology, immunity, reproduction, and fat body function [31]. Insect PLA_2 components of venom have been shown to cause pathologies such as anaphylaxis by eliciting cellular membrane disruption, inflammation, cellular necrosis, apoptosis induction, neurotoxicity, and hemolysis [32]. PLA_2 function is characterized by the ability to cleave cellular, non-cellular, and exogenous phospholipids to generate the eicosanoid precursor arachidonic acid (AA) along with saturated, monounsaturated, and polyunsaturated fatty acids (PUFAs) [33, 34]. PUFAs generated include ω-3 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), both of which are precursors of anti-inflammatory lipid mediators [35, 36]. Free AA produced by PLA2s are oxygenated by cyclooxygenase (COX) to yield prostaglandins (PGs), and by lipoxygenases (LOX) to yield leukotrienes (LTs). Cytochrome P450 monooxygenase can also change a double bond in AA to an epoxide, leading to the production of epoxyeicosatrienoic acids (EETs) [37]. Most terrestrial insects, however, lack AA-derived PUFAs, as their endogenous PLA2s cleave linoleic acid (LA) which can be converted to AA by desaturases and long chain fatty acid elongase [38, 39]. The newly formed AA can then undergo further conversion to a PGH_2, which possesses a five membered ring structure that is characteristic of PGs, before further conversion to PGs *via* cell specific enzymes. It has been reported that AA is not converted to PGH_2 by COX in insects. It is instead converted to PGH_2 *via* an insect peroxidase called peroxinectin [40, 41]. PGH_2 is then converted into cell specific PGs *via* cell specific enzymes, such as PGE_2 synthase converting PGH_2 into PGE_2 [42].

PGs are involved multiple physiological roles in insects such as eggshell production, signaling of actin remodeling, regulation of actin bundle formation during oogenesis in *Drosophila,* and regulation of fascin localization to the nucleus [43–45]. PGs play crucial roles in immune responses in insects by mediating the activation of hemocyte-spreading behavior involved in phagocytosis, nodulation, and encapsulation [37, 46]. This study characterizes the immunomodulatory effects of Sc-sPLA$_2$ (gene L596_023809) on *D. melanogaster* against bacterial infection. Survival and bacterial proliferation were assessed after a one-time coinjection of Sc-sPLA$_2$ and bacteria. Toxicity of the protein was also measured by administering a one-time dose of Sc-sPLA$_2$ to *D. melanogaster*. To understand the mechanisms contributing to immunosuppression, readouts of downstream immune responses were assessed, including AMP production, PO activity, and phagocytosis. Metabolomic analyses were conducted on hemolymph of flies treated with Sc-sPLA$_2$ to screen for changes in lipid metabolite and fatty acid composition. A cell lysis assay was used to determine whether toxicity was linked to lysis of host cell membranes, and hemocyte perfusion was performed to see if hemocyte circulation was affected by treatment with Sc-sPLA$_2$.

RESULTS

Sc-sPLA$_2$ has a toxic and immunomodulatory effect

An enzymatic assay was used to quantify the biological activity of recombinantly expressed Sc-sPLA$_2$ and a catalytically inactive mutant Sc-sPLA$_2$ (HH82-83QQ). Each protein was tested with a Red/Green BODIPY labeled phosphatidylcholine (PC)

substrate, and fluorescence emission intensity was measured and reported as a ratiometric value. The mutant $sPLA_2$ displayed significantly less activity than the wild type with a fluorescent 515/575 ratio close to 0, while the wild type displayed a fluorescent 515/575 ratio of over 1.5 (Figure 1A). Prior to assessing potential immunomodulatory phenotypes, a dose response for potential toxicity of Sc-$sPLA_2$ was measured. Toxicity increased as the dose increased where 5 ng of Sc-sPLA2 elicited minimal toxicity with over 90% survival rate by day 5, while 40 ng showed only a 65% survival rate in the same time frame (Figure 1B). Denatured protein displayed no toxicity throughout the 20-day period post injection, while all doses of Sc-$sPLA_2$ had an increase in toxicity after day 15 post injection (Figure 1B). To determine if the toxicity was linked to cell lysis, a cell lysis assay was performed with *D. melanogaster* Schneider 2 (S2) cells. Cells were treated with Sc-$sPLA_2$ or bee venom $sPLA_2$, which was screened for activity prior to experimentation to confirm enzymatic function (Supplementary Figure 3). The Sc-$sPLA_2$ did not cause cell lysis, while the bee venom PLA_2 showed an increase in cell lysis by significantly reducing the amount of live cells (8% reduction), showing that our findings are consistent with previous reports (Figure 1C) [47]. After evaluation of toxicity, each dose of Sc-$sPLA_2$ was then coinjected with 2,000 cells *S.p.* (LD_{10}) where we observed a significant reduction in survival after a one-time dose over the course of 20 days (Figure 2A). Sc-$sPLA_2$ significantly reduced the survival rate at each dose with the highest reduction observed at a dose of 40 ng which displayed a survival rate of only 20% after day 1 (Figure 2A). Microbial growth was also measured 24 hours post coinjection. We observed a significant increase in microbial load, approximately a 10-

fold increase at 40 ng (Figure 2B). The mutant Sc-sPLA$_2$ (HH82-83QQ) showed no change to the survival of the flies during coinjection (Supplementary Figure 1), confirming that the enzymatic activity of Sc-sPLA$_2$ was responsible for the immunomodulatory phenotypes observed.

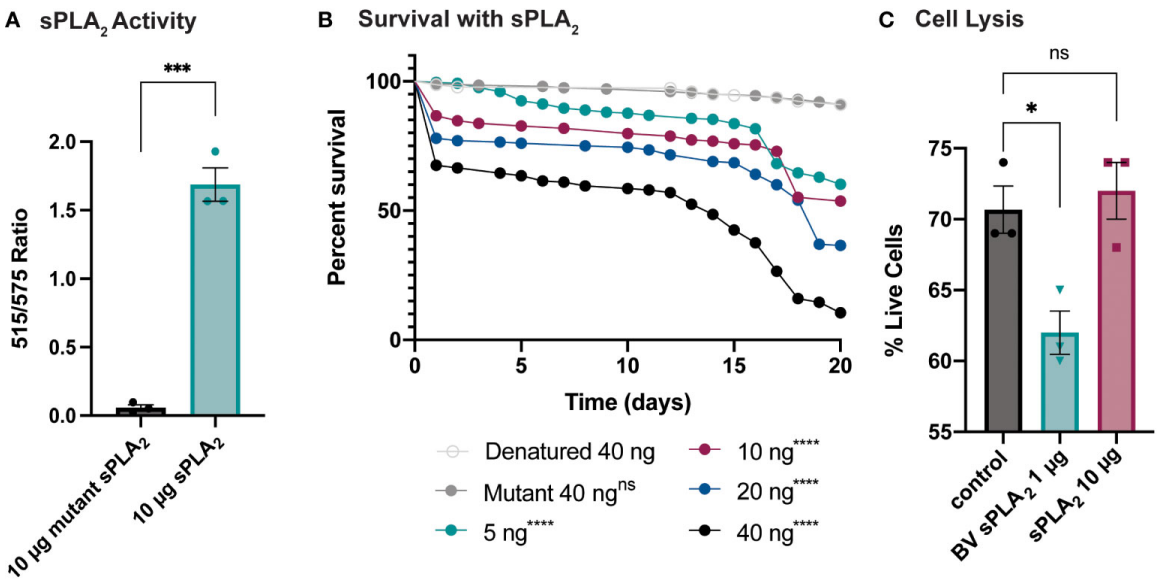


Figure 1.1 Panel for activity, survival, and cell lysis with Sc-sPLA$_2$. Survival rate of sPLA$_2$-only injected flies shows a dose-dependent toxic effect not caused by cell lysis. **(A)** *In vitro* activity data of Sc-sPLA$_2$ and mutant Sc-sPLA$_2$ (HH82-83QQ) at 10 µg each. Fluorescent emission intensity was measured at 515 and 575 nm and recorded as a ratiometric value. Negative control was subtracted as background from both absorbance values before calculating the ratio. Substrate used was a Red/Green Bodipy labeled PC. Experiment was done in triplicate. Statistics shown as unpaired t-test, error bars depict mean with SEM, p=0.0002, 4 degrees of freedom, n=3. **(B)** To measure the toxicity of the *S. carpocapsae* sPLA$_2$, 5–7-day old male flies were injected with various concentrations of protein and their survival was monitored for 20 days. Denatured protein shows no toxicity, and the intact protein shows a dose-dependent toxic effect with 40 ng showing the most significant toxicity. Survival curves n≥180. Log-rank test p-value significance compared to denatured 40 ng indicated by asterisks on Kaplan Meier graphs. 5, 10, 20, and 40 ng p<0.0001. Median survival is undefined for denatured, mutant, 5, and 10 ng, 19 days for 20 ng, and 14 days for 40 ng. **(C)** Quantification of cell lysis was measured by % of live cells after staining with a Bio-Rad TC20 automated cell counter. Sc-sPLA$_2$ showed no significant changes to the % of live cells, while bee venom sPLA$_2$ had a significant reduction which indicated an increase in cell lysis. Reactions were done in triplicate. Statistics shown as ordinary one-way ANOVA with Dunnett's multiple comparisons test p=0.0222, error bars depict mean with SEM, 8 degrees of freedom, n=3. All raw data available in Supplemental Materials. ns, not significant.

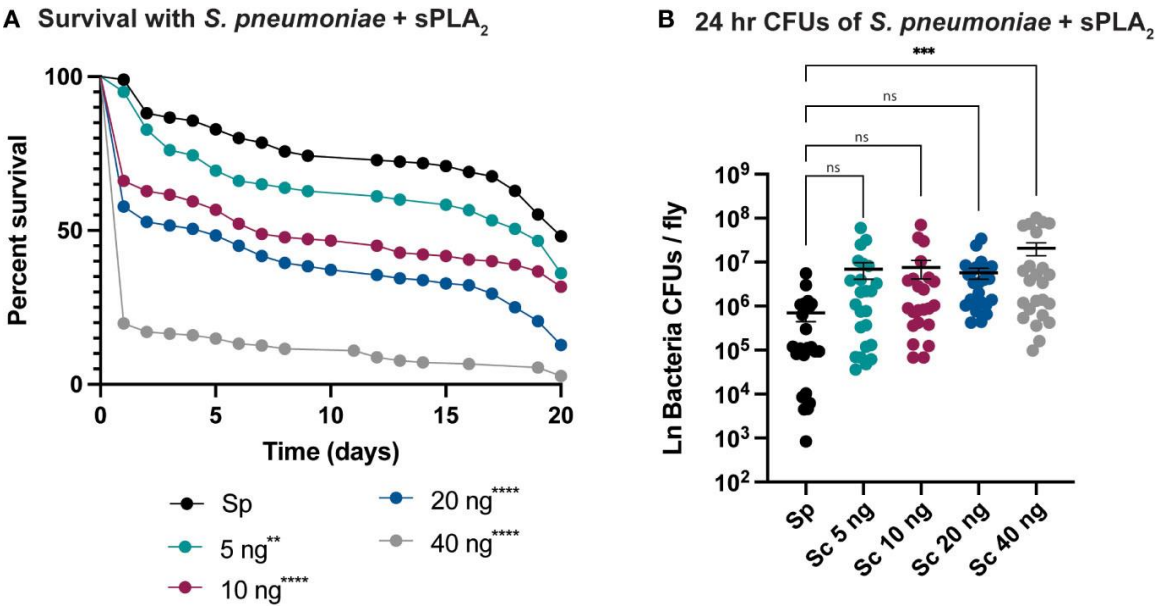


Figure 1.2 Bacterial challenge for survival and cfu assay. Sc-sPLA$_2$ elicits a dose-dependent immunomodulatory effect on survival and 24-hour CFUs in *Streptococcus pneumoniae* and sPLA$_2$ coinjections. **(A, B)** 5–7-day old male flies were coinjected with 2,000 cells of *S.p.* and various nanogram doses of sPLA$_2$. **(A)** Their survival was monitored for 20 days, showing a significant reduction in the outcome of survival in all doses compared to the *S.p.* only injected flies. Log-rank test p-value significance indicated by asterisks on Kaplan Meier graphs, n≥180. All doses were compared to *S.p.*-only dose, 5 ng p=0.0067, 10, 20, and 40 ng p<0.0001. Median survival of 20 days for *S.p.*-only, 17 days for 5 ng, 7 days for 10 ng, 5 days for 20 ng, and 1 day for 40 ng. **(B)** CFUs were measured 24-hours after injection and the 40 ng dose shows a significant increase in microbe load compared to the *S.p.*-only control group. Statistics shown as ordinary one-way ANOVA with Dunnett's multiple comparisons test p=0.0008 with 118 degrees of freedom. Error bars show mean+SEM, n≥24. When compared to the 40 ng dose, the 5 and 10 ng doses were not significant while 20 ng was significantly lower (p=0.0370). All raw data available in Supplemental Materials. ns, not significant.

Sc-sPLA$_2$ suppresses specific downstream immune responses

To better understand how Sc-sPLA$_2$ modulates immunity, we evaluated several readouts of immunity including PO activity and AMP production. PO activity serves as a catalyst for melanization. Flies were coinjected with Sc-sPLA2 and *Listeria monocytogenes*, a bacterium that elicits a robust disseminated melanization phenotype, to measure any changes to PO activity [24, 48]. Treatment with Sc-sPLA$_2$ showed no significant changes to PO activity compared to the Listeria-only group (Figure 3A). To further evaluate specific downstream immune responses, AMP production was measured 24-hours

postinjection. The protein treatment significantly reduced expression of *defensin* (Imd), *metchnikowin* (Imd), *diptericin* (Toll), and *drosomycin* (Toll), suggesting a suppressive effect on the Toll and Imd pathways (Figure 3B) [27, 28]. Phagocytosis is another important downstream immune process that is regulated by the cellular branch in insect immunity [20, 21]. Phagocytic activity in *D. melanogaster* was visualized and quantified *via* injection of fluorescently labeled conjugates of *E. coli* that fluoresce after exposure to the lysosome's low pH environment. These conjugates were coinjected with Sc-sPLA$_2$ to assess any changes in phagocytosis. We found that a one-time dose of 40 ng of Sc-sPLA$_2$ was able to significantly decrease phagocytosis activity 1-hour post injection (Figures 3C, D). Flies with the 40 ng dose had an average CTF of about $2.5*10^7$, while the negative control had a CTF of about $3.3*10^7$. 5 ng and 10 ng doses showed no significant effect on fluorescent change (Figure 3E). We evaluated if Sc-sPLA$_2$ targeted circulating hemocytes by measuring hemocyte concentration 1-hour post injection with enzyme. Flies injected with 40 ng of Sc-sPLA$_2$ had an average hemocyte concentration of 20 cells/µl. The negative control group (PBS) had an average concentration of 28 cells/µl, and the positive control group (20 ng bee venom sPLA$_2$) had an average concentration of 14 cells/µl (Figure 3F). This result ultimately shows that Sc-sPLA$_2$ is having a suppressive effect on the cellular and humoral responses of *D. melanogaster* immunity by targeting circulating hemocytes.

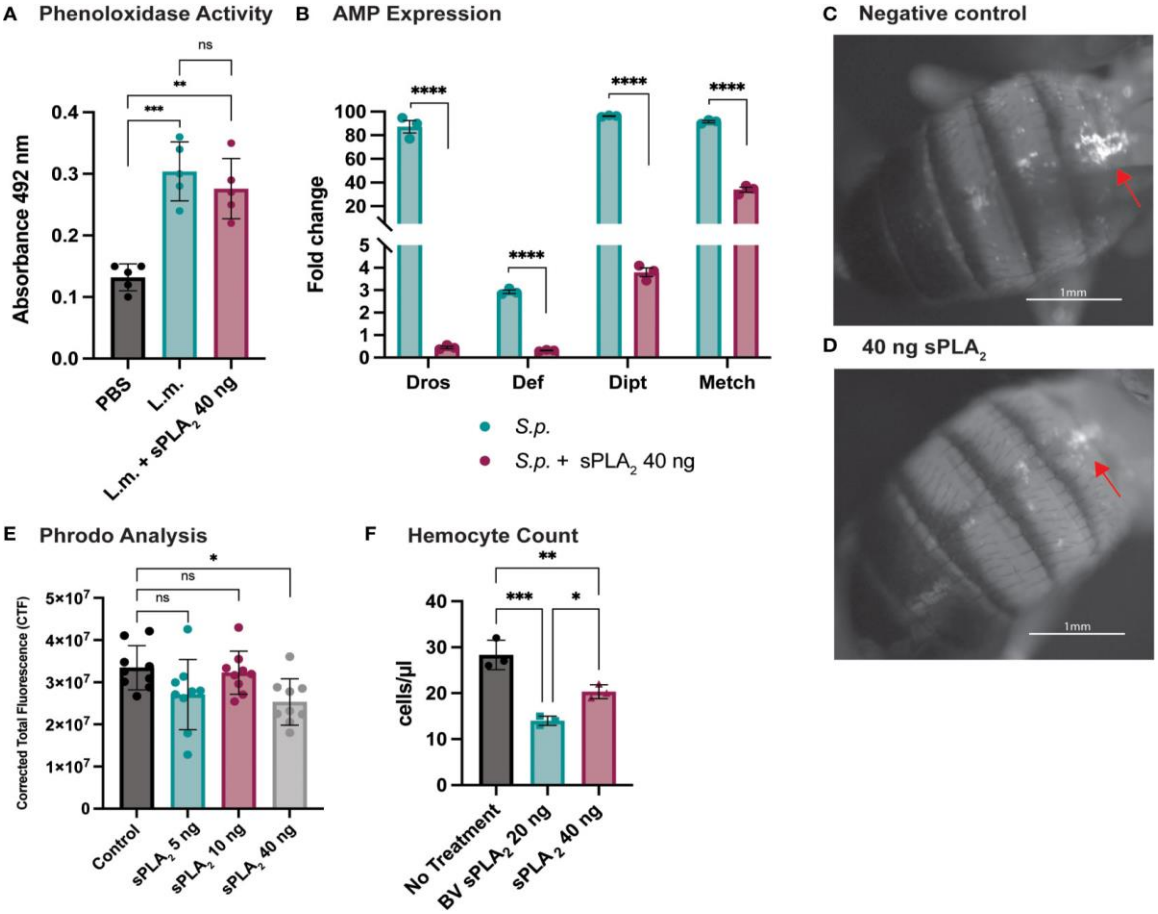


Figure 1.3 Downstream immune response assays. Specific downstream immune responses are affected by Sc-sPLA$_2$. **(A)** Phenoloxidase activity was measured 6 hours after injection with either PBS control, 10,000 cells *Listeria monocytogenes*, a known melanizer, or *L.m.* plus protein. An increase in PO activity was observed in the bacteria injected group but was not altered by the presence of protein. Experiments were completed 5 times with 30 flies in each treatment group. Statistics shown as ordinary one-way ANOVA with Dunnett's multiple comparisons test *L.m.* p<0.0001, 40 ng p=0.0003 with 14 degrees of freedom, error bars depict mean with SEM. **(B)** Antimicrobial peptide production was measured by quantitative PCR 24 hours after injection with *S.p.* or *S.p.* plus protein. Four different AMPs were measured*, Drosomycin* (Toll), *Defensin* (Imd), *Diptericin* (Imd), and *Metchnikowin* (Toll) were all decreased after protein injection. Statistics shown as 2-way ANOVA with a Tukey multiple comparisons test, p<0.0001 for all sets with 16 degrees of freedom. Experiments repeated 3 times with 15 flies in each group. **(C)** Phagocytic activity was measured with the pHrodo assay showing fluorescence once phagocytosed. pHrodo only injected flies show higher amounts of fluorescence. **(D)** Fluorescence is decreased in flies injected with 40 ng of sPLA2 protein. Representative images are depicted. **(E)** We found the 40 ng dose of sPLA2 protein to significantly reduce phagocytosis one hour post injection. The 5 and 10 ng doses are not significantly different from the 40 ng sPLA2 dose. Statistics shown as ordinary one-way ANOVA with Dunnett's multiple comparisons test p=0.0243 with 35 degrees of freedom. Experiments were repeated 3 times with 3 flies per group. **(F)** We found that 40 ng dose of sPLA2 protein had a significant reduction of circulating hemocytes one hour post injection. Each experiment was repeated 3 times with 10 flies in each group for every treatment. Statistics shown as ordinary one-way ANOVA with Tukey's multiple comparisons test. Asterisks indicating the following p-value cut offs: 0.05-0.033*, 0.033-0.002**, 0.002-0.0001*** and <0.0001****. ns, not significant.

Sc-sPLA$_2$ displays exponentially higher activity with PLPE and AA

To better understand the effect of Sc-sPLA$_2$ on lipid metabolism and which phospholipid sources were a preferred target for this enzyme, we utilized lipidomics by performing a high-throughput mass spectrometric based assay [49]. This assay revealed the *in vitro* activity of Sc-sPLA$_2$ towards both natural and synthetic membrane phospholipids in mixed micelles. First, we explored preference of Sc-PLA$_2$ for phospholipid head group by using four major phospholipid head groups for the *sn-3* position which included phosphoethanolamine (PE), phosphoserine (PS), phosphoglycerol (PG), and phosphocholine (PC). For the *sn-1* position we utilized palmitic acid due to its ability to be produced *de novo* in *Drosophila* [50]. Previous studies showed that the *sn-1* fatty acid did not affect the activity of PLA2s and thus we did not conduct optimization for that position [49]. For optimization and head group studies we utilized linoleic acid (LA) at the *sn-2* position since it is the most abundant PUFA in *D. melanogaster* [50]. Reactions were run for 30 minutes as it was determined in previous studies to be the most optimal time for multiple PLA$_2$s [49]. Surfactant concentration and enzyme concentration optimization reactions were conducted to determine the conditions to use for downstream experimentation (Supplementary Figure 2). For determining Sc-sPLA$_2$ preference for phospholipid head groups, we used the lipid substrate 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphox where "x" represents one of the four major lipid head groups. Thus, the lipid headgroup substrates utilized for the reaction were PLPE, PLPS, PLPG and PLPC. The experiment showed that activity towards PLPE by Sc-sPLA$_2$ was exponentially higher

than all other headgroups (Figures 4A, B). Interestingly, PE abundance has been linked to Toll pathway expression in *D. melanogaster* [51]. We performed subsequent experiments using lipid substrates with the PE headgroup to determine Sc-sPLA$_2$ fatty acid preference at the *sn-2* position. The fatty acids used were oleic (OA), LA, and arachidonic acid (AA). OA was selected as it is an 18-carbon fatty acid with high abundance in *D. melanogaster* and can be converted to LA [52, 53]. LA and AA are precursors to immunomodulating eicosanoids with AA being the most common precursor in mammals [33, 36, 54]. We found that Sc-sPLA$_2$ displayed high activity to both LA and AA in comparison to OA (Figure 4C). Overall, these data illustrate that Sc-sPLA$_2$ displays high activity with lipid species that have downstream effects on immunity.

Figure 4.

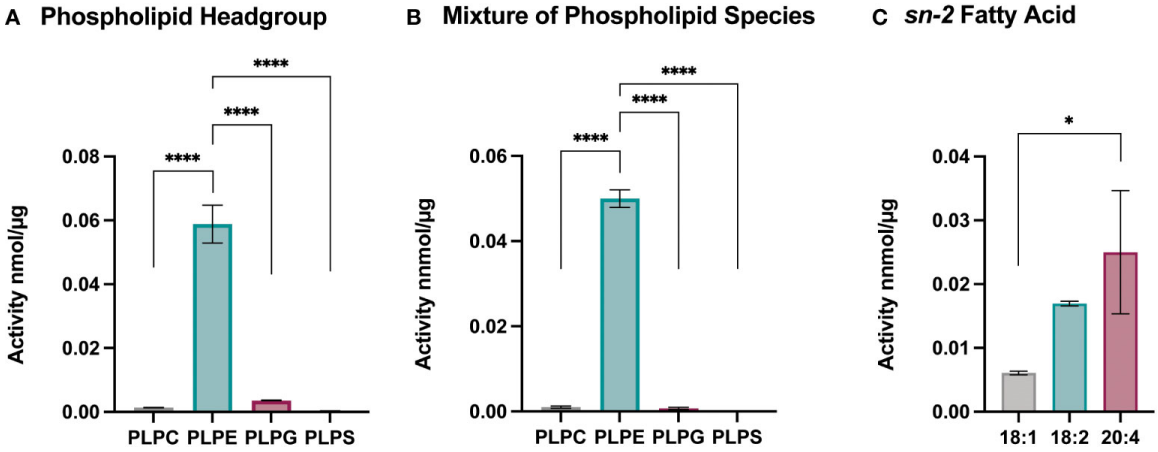


Figure 1.4 Activity with various lipid substrates. Enzymatic activity assays show sPLA$_2$ prefers PLPE. **(A)** Enzymatic activity of Sc-sPLA$_2$ towards 100 µM of PLPC, PLPE, PLPG and PLPS. Statistics shown as ordinary one-way ANOVA with Tukey's multiple comparisons test. PLPC vs PLPE, PLPE vs. PLPG, and PLPE vs. PLPS $p<0.0001$ with 11 degrees of freedom. **(B)** Enzymatic activity of Sc-sPLA$_2$ towards 100 µM mixture (20 µM each) of PLPC, PLPE, PLPG and PLPS. Statistics shown as ordinary one-way ANOVA with Tukey's multiple comparisons test. PLPC vs PLPE, PLPE vs. PLPG, and PLPE vs. PLPS $p<0.0001$ with 11 degrees of freedom. **(C)** Enzymatic activity of Sc-sPLA$_2$ towards 100 µM PLPE species with an *sn*-2 fatty acid position of OA (18:1), LA (18:2), and AA (20:4). Statistics shown as ordinary one-way ANOVA with Tukey's multiple comparisons test. 18:1 vs. 20:4 $p=0.0142$ with 8 degrees of freedom. Negative control values were subtracted from each reaction. Experiments were done in triplicate. Error bars depict mean with SEM.

Sc-sPLA2 alters fatty acid composition *in vivo*

To further elucidate the underlying molecular mechanisms of Sc-sPLA$_2$'s immunomodulatory effects, we used mass spectrometry to analyze the hemolymph of protein-injected flies. A targeted approach allowed for identification of known fatty acids and lipid metabolites that were altered after treatment with the protein. sPLA$_2$ activity produces lipids that can have roles as immune mediators and thus we anticipated this experiment would identify fatty acids and lipid metabolites with a significant role in insect immunity [30]. The lipid panel for the mass spectrometry analysis consisted of 33 lipids. 24 fatty acids were detected in the fly hemolymph, including C20, C22, C23, C24, C26 fatty acids (Figure 5). When comparing the Sc-sPLA$_2$ treatment group to the PBS control, we observed an increase in LA, OA, and palmitoleic acid (PA-16:1), with a decrease in myristic acid (MA-14:0). When compared to the mutant control group, Sc-sPLA$_2$ elicited the same significant changes except it did not significantly change PA, and no significant changes in lipid profiles between the PBS and mutant control groups were observed (Figure 5). Out of the downstream oxylipin metabolite library used for further analysis we saw significant abundance of 17 different lipid metabolites, with Sc-sPLA$_2$ causing a significant reduction of 9, (10)-, and 12, (13)-EpOME at 12-hours post-injection (Supplementary Figure 4).

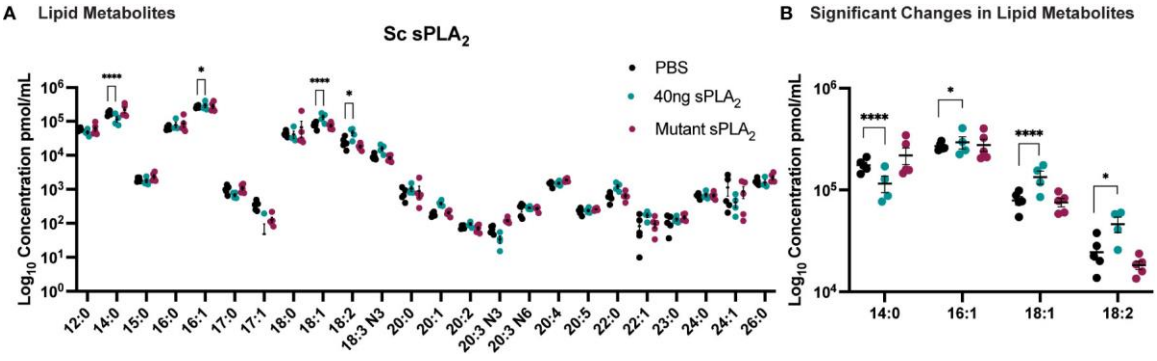


Figure 1.5 Fly hemolymph lipid metabolite panel. Injection of 40 ng recombinant Sc-sPLA$_2$ induced significant changes in lipid metabolites. **(A)** Lipid panel consisted of 33 fatty acids but only 24 were detected in the fly hemolymph samples. **(B)** A reduction of myristic acid (MA-14:0) compared to both PBS ($p<0.0001$) and the mutant control ($p<0.0001$). Sc-sPLA$_2$ generated more oleic acid (OA-18:1) compared to both PBS and the mutant control (to PBS $p<0.0001$, to mutant $p=0.0023$), and linoleic acid (LA -18:2) compared to PBS ($p=0.0414$) and increased palmitoleic acid (PA-16:1) in comparison to the PBS control ($p=0.0256$). There were no differences between the mutant and PBS control groups. Experiments were repeated 5 times with 200 flies per treatment group. Error bars show mean + SEM with statistics shown as multiple unpaired t-tests.

DISCUSSION

It has been well established that sPLA$_2$ activity plays an important role in immune response by cleaving PUFAs such as AA from glycerophospholipids resulting in production of downstream immunomodulatory eicosanoids [33, 34]. While this process is well defined in mammals, the presence of lipid signaling in insect immunity has not been validated and has even been disputed due to their lack of C20 and C22 PUFAs necessary for eicosanoid production [55]. It has been recently reported however, that insects are able to generate eicosanoids and their precursor AA by converting cleaved LA into AA for downstream eicosanoid production [38, 54]. With a potential mechanism in place for lipid signaling mediated immunity in insects, we evaluated the role of an sPLA$_2$ from a parasitic nematode, Sc-sPLA$_2$, in host immunomodulation to bacterial infections in *D. melanogaster*.

We hypothesized that Sc-sPLA$_2$ would display immunosuppressive effects on its host since it is secreted by *S. carpocapsae* infective juveniles (IJs) during infection. sPLA$_2$ enzymes are notable for eliciting immunostimulatory responses *via* downstream production of proinflammatory eicosanoids from arachidonic acid [33]. However, they are also able to cleave PUFAs such as EPA and DHA which are then converted to downstream anti-inflammatory mediators, indicating that sPLA$_2$ enzymes can also have immunosuppressive capabilities [33]. In addition to immunomodulatory effects, PLA$_2$ enzymes have been reported to display toxic effects on hosts. This is facilitated by necrotic cell lysis *via* enzymatic cleavage of the phospholipid cell membrane by PLA$_2$s, resulting in loss of cell membrane integrity and release of cellular components [47, 56]. Sc-sPLA$_2$ was able to display a dose dependent toxic effect in *D. melanogaster* at a low dose of Sc-sPLA$_2$ (5 ng). These flies had around a 95% survival rate by day five in comparison to the higher dose (40 ng) that displayed a 65% survival rate. After day five toxicity had a slow increase for all doses up until day 15 where another notable increase in toxicity occurred resulting in decreased survival rates. This highlighted that the enzyme's toxic effects on the host were both time- and dose-dependent. We found no significant change in the amount of cell lysis of S2 cells in comparison to the negative control, indicating that the toxic effects are not caused by cell lysis, but perhaps because of other cell death mechanisms such as apoptosis and necroptosis. The fact that Sc-sPLA$_2$ elicits an immunosuppressive phenotype at a 5 ng dose shows significant importance as this is a physiologically relevant dose. Twenty IJs of *S. carpocapsae* secrete approximately 10 ng of crude ESPs in 24 hours [29]. The ES protein

composition of *S. carpocapsae* is approximately 500 proteins, with Sc-sPLA$_2$ being just one component. With enough IJs however, and the mixture of multiple immunomodulatory proteins, it is likely that Sc-sPLA$_2$ aids in overcoming the host immune response in a natural infection.

We evaluated the effects of Sc-sPLA$_2$ on downstream immunity in the fly. Fly immunity starts with pathogen specific recognition by the toll and imd pathways, which then leads to either a cellular immune response by specialized hemocytes, or a humoral immune response *via* production of Toll- or Imd-specific AMPs secreted from the fat body [19, 26]. Melanization is independent of the Toll and Imd pathways and is dependent on the proPO-PO cascade [22, 23]. Our findings showed that Sc-sPLA$_2$ had no effect on PO activity but caused a reduction in the expression of the AMPs *Metchnikowin, Diptericin, Defensin* and *Drosomycin* and significantly reduced phagocytosis at a one-time dose of 40 ng. There was not a significant effect on phagocytosis at 5 and 10 ng (Figure 3E). We speculate that this was due to the time point for observing fluorescence of the assay being too early for the lower doses to generate a significant difference. Methods for the pHrodo Red *E. coli* BioParticles conjugate state that fluorescence can be observed after 30-60 minutes, with many experiments opting to observe fluorescence generally after 2 hours. To further evaluate how Sc-sPLA$_2$ specifically elicited these immunomodulatory phenotypes, we measured hemocyte circulation 1 hour post injection with 40 ng of Sc-sPLA$_2$ and found a significant reduction in hemocyte concentration. This highlighted that the enzyme did in fact trigger cell death. We opted to observe effects one hour post injection to see how early introduction of the sPLA$_2$ begins to affect the immune

response. The effects at this early time point also reinforce that at least some immunosuppressive effects are observed prior to lethal toxicity as flies are still all alive at one hour post injection. Overall, these findings suggest that the Sc-sPLA$_2$ suppresses both the Toll and Imd pathways along with cellular immune responses by targeting circulating hemocytes.

We utilized lipidomics for a mass spectrometry-based high-throughput assay to determine the preference of Sc-sPLA$_2$ for specific lipid targets *in vitro*. These data would provide some insight regarding the lipids Sc-sPLA$_2$ may target during a natural infection by *S. carpocapsae* in insects. Our data showed that Sc-sPLA$_2$ had exponentially higher activity with PE as a substrate than the other lipid headgroups. This is significant as PE is the most abundant phospholipid for cellular membranes in *D. melanogaster* [51, 57]. Displaying significant catalytic activity against PE lipid substrates is likely advantageous for Sc-sPLA$_2$ in terms of modulating insect immunity. It is important to note that the Enzchek Bee venom sPLA$_2$ also displayed its highest levels of activity with the PE substrate as opposed to PC (Supplementary Figure 3). The Enzchek activity kit utilizes PC as the commercial substrate, but our experiments show that it could be more advantageous to use PE as the substrate for commercial activity kits. We also used the novel mass spectrometry-based high-throughput assay to determine preference *in vitro* for fatty acid side chains at the sn-2 position. The *in vitro* assay showed that Sc-sPLA$_2$ displayed highest activity with LA and AA at the *sn-2* position. Activity against OA was measurable but noticeably lower than activity against LA and AA. It is strategic for Sc-sPLA$_2$ to display significant activity with OA as it can be converted to the

eicosanoid precursor LA [52]. Linoleic acid is the most abundant PUFA in insects such as *D. melanogaster* and like AA, it is a precursor to generating eicosanoids involved in downstream immune responses [36, 50]. Arachidonic acid can generate pro-inflammatory eicosanoids in its omega-6 form, and anti-inflammatory eicosanoids in its omega-3 form. The higher activity levels displayed against these two PUFAs illustrates that Sc-sPLA$_2$ could potentially be suppressing immunity by generating downstream immunosuppressive lipid metabolites, or by interfering with host endogenous sPLA$_2$ activity. Little is known about endogenous sPLA$_2$ activity in fly immunity. We collected fly hemolymph after injection of Sc-sPLA$_2$ to perform mass spectrometry to determine fatty acid and lipid metabolite composition 12-hours postinjection. Fatty acid composition postinjection showed an increase in PA, OA, and LA, and a decrease in MA. An increase in OA and LA is consistent with the high activity levels that Sc-sPLA$_2$ displayed against phospholipids with these fatty acid side chains. Sc-sPLA$_2$ increasing PA in the hemolymph may play a role in the downstream immunosuppressive phenotypes observed, as increased PA was shown to elicit anti-inflammatory effects in animal models [58]. Our data showed there was an increase in PA by Sc-sPLA$_2$ when compared to the PBS control, but there were no significant changes in PA compared to the mutant control group. A likely explanation is that the 12-hour time point was long enough for the low activity of the mutant to generate enough PA to affect the comparison. The mutant enzyme had low enough activity not demonstrate any immunomodulatory phenotypes but was still able to have low activity levels detected by the Enzchek activity assay. The data also showed a decrease in free

MA that could be linked to the high activity demonstrated to the major lipid headgroup PE by Sc-sPLA$_2$. PE is 50% of cellular membrane phospholipids in *D. melanogaster*, and MA is utilized to anchor proteins to the cellular membrane [57, 59]. It is possible that disruption of the cellular membrane *via* cleavage of PE, could lead to free MA to be utilized in myristoylation and sidechain palmitoylation to anchor more proteins to the disrupted membrane for preserving stability and cellular function. This would then result in a reduction of free MA in the hemolymph. Myristic acid is known to play a direct role in two classes of protein fatty acid acylation: N-terminal myristoylation and side-chain palmitoylation [60]. This promotes anchoring of proteins to the cell membrane [59]. The protein substrates that are products of acylation carried out by Myristoyl–CoA: protein N-myristoyltransferase (NMT) include those that are key components of intracellular signaling [61]. Palmitic acid has direct impact in immunity as it has been shown in animal models to decrease expression of proinflammatory markers and adipokines [58, 62–64]. Palmitic acid suppresses the expression of monocyte chemoattractant protein 1 (MCP-1) and TNF-α in adipose tissue suggesting the lipid has downstream anti-inflammatory effects [62, 64]. The fatty acids changed by Sc-sPLA$_2$ each demonstrate a role in the immune response *via* lipid signaling or potential other downstream mechanisms, thus implicating several pathways the enzyme could be interfering with to suppress immunity other than hemocyte reduction. In addition to assessing fatty acid composition, we also analyzed the hemolymph for any downstream changes to lipid metabolite composition. Findings showed that Sc-sPLA$_2$ treated flies had a reduction in 9,(10)-EpOME and 12,(13)-EpOME. These lipid metabolites are synthesized by activated

neutrophils in mammals and are known low-level stimulators of respiratory burst, a process that occurs during phagocytosis [65–67]. LA is a potent inducer of respiratory burst but is increased in the hemolymph after enzyme treatment [67]. Soluble metabolites from epoxide hydrolase (DiHOMEs) are also directly responsible for respiratory burst inhibition, but there is no change in DiHOMEs after enzyme treatment [67]. This information combined with previous studies that showed 9,(10)-EpOME and 12,(13)-EpOME to suppress immune response in *Spodoptera exigua*, make it likely their reduction by Sc-sPLA$_2$ is a byproduct from the enzyme triggering cell death of hemocytes in the hemolymph [68]. Overall, these data suggest that the molecular effects underlying sPLA$_2$ suppression of the cellular immune response is *via* targeting of circulating hemocytes. Future studies on how Sc-sPLA$_2$ directly affects hemocyte proliferation and morphology can help further elucidate mechanistically how the enzyme is reducing hemocyte circulation.

In summary this study showed that Sc-sPLA$_2$ experimentally dampened the immunity of *D. melanogaster* by suppression of phagocytosis and both the toll and imd pathways. In addition to immunomodulation, Sc-sPLA$_2$ also displayed dose-dependent toxicity to the host that was not elicited by cell lysis. We hypothesize that the lipids being cleaved by the PLA$_2$ enzyme are from hemocytes which disrupts their ability to recognize and phagocytize cells, while producing a toxic molecular product to the host. The change in the lipid composition as a result of this process could also be disrupting the lipid signaling processes, leading to further suppression in immunity such as reduced toll and imd activation. Further elucidating the specificity of the molecular mechanisms affected

by Sc-sPLA$_2$ can continue to validate the presence of lipid signaling in *D. melanogaster* immunity, which would improve the tools available for biomedical research and further enhance the translation of fly research in addressing inflammatory and infectious diseases.

METHODS

Plasmid construction

A 414 -bp DNA fragment of Sc-sPLA$_2$ gene L596_023809 was amplified by PCR using primers 5' –ACCATCATCACCACAGCCAGGGCAAACTTATCAAGAAGAATGTCG – 3' (forward primer) and 5' – TTAAGCATTATGCGGCCGCATTACGCGTGGAAATCGAGC – 3' (reverse primer) in which a *Bam*HI site at the 5′ end and a *Hind*III site at the 3′ end was introduced for cloning it into a pETDuet-1 vector. The mutant Sc-sPLA$_2$(HH82-83QQ) had two histidine amino acid sequences at positions 82 and 83, mutated to glutamine. The mutant was synthesized, optimized and inserted into a pETDuet-1 vector utilizing a *Bam*HI site at the 5′ end and a *Hind*III site at the 3′ position. The mutant construct was generated by Bio Basic Inc.

Recombinant protein expression and purification

Sc-sPLA$_2$ and mutant Sc-sPLA$_2$ (HH82-83QQ) were recombinantly expressed using *E. coli* BL21 DE3 cells in LB media for 24 hours after induction with IPTG. Sc-sPLA$_2$ was purified from inclusion bodies with Thermo Scientific™ HisPur™ Ni-NTA Resin *via* gravity filtraticon. The protein was refolded with a 24-hour dialysis against a

20 mM Tris, 1.0 M Urea, 300 mM NaCl, and 5% glycerol pH 8.0 buffer. After refolding the protein was dialyzed once more for 24 hours and stored in a 20 mM Tris, 300 mM NaCl, and 5% glycerol pH 8.0 buffer. Mutant Sc-sPLA$_2$ (HH82-83QQ) was first purified with Thermo Scientific™ HisPur™ Ni-NTA Resin *via* gravity filtration. The protein was dialyzed against a 20 mM Tris pH 8.0 buffer for 24 hours. Further purification was conducted with FPLC using a Mono Q™ anion exchange column, after which the protein was isolated using size exclusion and stored in a 20 mM Tris, 300 mM NaCl, and 5% glycerol pH 8.0 buffer. Both Sc-sPLA$_2$ and mutant Sc-sPLA$_2$ (HH82-83QQ) presence were confirmed using SDS-PAGE. Concentrations were measured using Invitrogen™ Qubit™ Protein and Protein Broad Range (BR) Assay Kits, and the proteins were flash frozen with liquid nitrogen and stored at −80°C.

Protein activity assay

Enzymatic activity of Sc-sPLA$_2$ and mutant Sc-sPLA$_2$ (HH82-83QQ) was assessed utilizing the EnzChek™ Phospholipase A2 Assay Kit. Each reaction contained 10 µg of protein and 50 µl 1.67 µM Red/Green BODIPY labeled phosphatidylcholine (PC) substrate for a total of 100 µl. Reaction time was 30 minutes at room temperature. Negative control was designated as buffer only plus the substrate. Emission intensity was measured at 515 and 575 nm with excitation at 460 nm, and the activity was recorded as a ratiometric value (515/575 nm). Negative control values at 515 and 575 nm were subtracted from the protein reactions before calculation of the activity ration. Reactions were triplicated.

Lipidomics mass spectrometry assay

To determine activity preference of Sc-sPLA$_2$ 1.0 ugs of the recombinantly expressed sPLA$_2$ proteins was added to reactions that contained 100 uM of PLPC, PLPA, PLPG, PLPE, or PLPS, 400 uM of surfactant, 2.5 uM of 17:0 LPC and reaction buffer (20 mM Tris and 5 mM CaCl2 buffer pH 8.0). The reaction buffer was used to store the lipids and surfactant. For mixed phospholipid head group reactions, we used the same conditions as previously described for the enzyme, 17:0 LPC, surfactant, and buffer. For the lipid substrate however, all head groups were combined for a total concentration of 100 uM (20 uM for each different phospholipid headgroup). Enzymatic reaction was performed in a 96 well-plate using a Benchmark Scientific H5000-H MultiTherm heating shaker for 30 min at 28°C. Negative control was reaction buffer only with no protein, and positive control was 0.125 ugs of Enzchek Bee Venom sPLA$_2$ to ensure the reaction is working as intended (these controls were used for all optimization and specificity reactions). Reactions were quenched with methanol/acetonitrile (80/20, v/v), and the samples were analyzed using the HPLC-MS system. Activity was reported as nmols/ug with subtraction of the negative control as background. Experiments were done in triplicate with error bars on the graph representing standard deviation. For determining activity preference for *sn-2* fatty acids, 1.0 ugs of the Sc-sPLA$_2$ was added to a reaction of 100 uM phospholipid substrate, 400 uM surfactant, and 2.5 uM of 17.0 LPC. The phospholipid substrate used the experimentally determined preferred phospholipid head group PE. Each lipid substrate had a different *sn-2* fatty acid of either LA, OA, or AA for a concentration of 100 uM in separate reactions. Enzymatic reaction was performed in a 96 well-plate using

a Benchmark Scientific H5000-H MultiTherm heating shaker for 30 min at 28°C. Negative control was reaction buffer only with no protein, and positive control was 0.125 ugs of Enzchek Bee Venom $sPLA_2$. Mass spectrometry analysis was conducted at the UC Riverside Core Facility. Experiments were done in triplicate with error bars on the graph representing standard deviation.

UCR core facility QQQ lipidomics method

The targeted analysis was performed using a QQQ XEVO TQ-XS (Waters Corp., Milford, MA, USA) at the UC Riverside Metabolomics Core. The liquid chromatography-mass spectrometry (LC-MS) autosampler was maintained at 4°C prior to analysis. For the analysis, an injection volume of 2 µL of the extract was used. The separation was performed on the Waters XSelect CSH Phenyl-Hexyl column (3.5 µm, 3.0 × 100 mm (Waters Corp., Milford, MA, USA). The flow rate was maintained at 0.8 mL/min at 30 C. Mobile phase A consisted of ACN/water (95/5, v/v, pH=8.0) containing 25 mM AcNH4 and Mobile Phase B consisted of ACN/water (50/50, v/v, pH=7.5) containing 25 mM AcNH4. The gradient separation method was used as follows: 8 min (0–0.2 min 99% B; 0.2-3.0 min 99% B to 1%B, 3.0-3.8 min 1% B; 3.8-3.9 min 1% B to 99% B; 3.9-8.0 min 99% B. The MS data were acquired in multiple reaction monitoring (MRM) mode. The electrospray ionization was performed in positive ion mode. The source and desolvation temperatures were maintained at 150°C and 600°C, respectively. The desolvation gas was set to 1100 L/h and cone gas to 150 L/hr and the collision gas was set to 0.15 mL/min. All gases used were nitrogen, other than the collision gas which was argon. The capillary voltage was 1.5 kV. The data was normalized for relative

abundance against the internal standard (LPC 17:0). Targeted data processing was performed with the open-source Skyline software [69].

Fly stock/maintenance

Oregon R flies were grown on D2 glucose medium from Archon Scientific (Durham, North Carolina) and kept at 25°C with 50% humidity on a 12h light 12h dark cycle.

Bacterial stock maintenance

Methods were adapted from [53]. *Streptococcus pneumoniae* was grown by shaking in glass vials with 5 mL tryptic soy (TS) broth (Difco TS broth, catalase, streptomycin) at 37°C with 5% CO_2 overnight. The overgrown culture was diluted in catalase (100 µL) and TS to yield a final volume of 20 mL in a flask and incubated shaking until the OD_{600} ~ 0.4 (about 1 hour). The culture was then diluted again to a final volume of 50 mL, with 150 µL catalase, and incubated until the OD_{600} ~ 0.2 - 0.4 (above 0.5 is no longer in log phase). 5% glycerol was added to the final culture and stored then in 1mL aliquots at -80°C. To use the aliquots, one tube was thawed, spun down at 18,000 g for 5 minutes, the supernatant was removed, and the pellet was resuspended in the desired amount of PBS (50 - 60 µL yields ~ 100,000 CFUs) and serially diluted to yield the appropriate CFU doses. For quantification of CFUs, *S.p.* was plated on TSA agar plates supplemented with 50 mL/L sheep's blood. *Listeria monocytogenes* (serotype 4b, 19115, (ATCC, VA)) was also grown in batches in brain heart infusion (BHI) medium at 37°C in aerobic condition. Cultures were grown overnight in a flask inoculated with a fresh colony and re-diluted under log phase (below OD_{600} ~ 0.2) and grown up to the desired OD_{600} (~0.4). The entire volume was transferred to a 50mL centrifuge tube for vortexing.

Before freezing, a 5% glycerol solution was added to the culture and 1mL aliquots were stored at -80°C. To use the aliquots, one tube was thawed, spun down at 18,000 g for 5 minutes, the supernatant was removed, and the pellet was resuspended in the desired amount of PBS (90 - 100 µL yields ~ 100,000 CFUs) and serially diluted to yield the appropriate CFU doses. For quantification of CFUs, *L.m.* was plated on BHI plates.

Fly injections, survival and CFUs

Methods were adapted from [53]. For injections and immune assays, 5-7-day-old male Oregon R flies were anesthetized with CO_2 and injected with various CFU doses yielding a total volume of 50 nL precisely using a MINJ-FLY high-speed pneumatic injector (Tritech Research, CA) and individually pulled calibrated glass needles. Flies were injected into the abdomen close to where the thorax meets and slightly ventral from the dorsal-ventral cuticle axis, easily visible below the haltere. Survival studies were carried out for all of the pathogens we tested. After injection of the CFU dose or phosphate buffered saline (PBS) control, flies were placed in vials in groups of 30 with a total of 60 flies per experimental or control group. Flies injected with the human pathogens (*S.p.* and *L.m.)* were kept at 28°C with 50% humidity. The number of dead flies was counted daily, and Kaplan-Meier survival curves were generated with GraphPad Prism software with statistics shown as log-rank analysis (Mantel-Cox). Survival experiments were at least triplicated. CFUs were determined by homogenizing a single infected, or buffer-injected fly in 200 µL of PBS, serially diluted and plated on the appropriate agar plates and incubated overnight. Colonies were counted the next day. At least five flies per condition were homogenized for CFU quantification each time an injection experiment

was done to measure time 0 CFUs which are representative of all fly strains. All treatment groups were injected at the same time for each experimental replicate. Using GraphPad Prism software, results are shown as scatter plots with statistical significance analyzed using an unpaired t-test.

Phenoloxidase activity

Methods adapted from [53]. Flies were injected with 10,000 CFUs of *L. monocytogenes* to elicit an immune induced melanization cascade. Phenoloxidase activity was measured as previously described [53]. To collect hemolymph, 20-30 flies 6 hours post injection (p.i.) were pricked through the thorax and placed in a pierced 0.5 µL Eppendorf tube and covered with glass beads, then placed inside a 1.5 µL Eppendorf tube containing 30 µL of PBS. Samples were centrifuged at 10,000 g for 20 minutes at 4°C. Using a clear 96-well plate, each well contained 160 µL L-Dopa (3 mg/mL) dissolved in phosphate buffer (37.5% 1 M potassium phosphate, 62.5% 1 M sodium phosphate, pH 6.5), 35 µL of hemolymph sample and 5 µL $CaCl_2$ (20 mM). PO activity was measured by kinetic reads at 29°C at 492 nm every minute for 120 min with 5 seconds of shaking between reads. The OD of a blank control was subtracted from all biological values. Experiments were replicated five times with three technical replicates per experiment. Data were plotted as mean+SEM by taking the peak OD value (timepoint ~ 60 min). Statistics shown as an unpaired t-tests done in GraphPad Prism.

Antimicrobial peptide gene expression - qPCR

Methods adapted from [53]. Total RNA was extracted from 15 *S. pneumonia* or *S.p.* plus recombinant protein injected flies 24 hours post-injection using Trizol reagent (Molecular

Research Center, Inc; Cincinnati, Ohio) according to the manufacturer instructions. Integrity of RNA was confirmed by observing bands on an agarose gel and concentration was determined by nanodrop. Reverse transcription of RNA was done using ProtoScript II First Strand cDNA synthesis kit (New England BioLabs, NE, E6560L) following the manufacturer protocol, in a MultiGene OptiMax Thermal Cycler (Labnet international, NJ). The qRT-PCR was done with a CFX Connect Bio-Rad system with Perfecta SYBR green supermix (QuantaBio, MA) and gene specific primers for *Defensin, Drosomycin*, *Diptericin, Metchnikowin* and the housekeeping gene *Tubulin* (Integrated DNA Technologies, IA). Cycling conditions for PCR included a denature step at 94 ˚C for 15 seconds, annealing step at 55 ˚C for 30 seconds, and an extension step at 68 ˚C for 1 minute. All steps were conducted for a total of 40 cycles. Fold change was measured according to the ΔΔCT Method. Experiments were carried out with three biological replicates with plots shown as bar graphs with individual points representing each replicate. Statistics shown as One-way ANOVA done in GraphPad Prism.

Cell culture maintenance

12 ml room temperature of fresh medium [500 mL Schneider's *Drosophila* medium (Thermo Fisher Scientific, #21720-024-500ML) (Store 4˚C) + 56 mL Fetal bovine serum (FBS; Thermo Fisher Scientific, #10082147, Store at -20˚C or 4˚C) +5.6 mL Penicillin-streptomycin solution (PSS: Thermo Fisher Scientific ®, Store at -20˚C or 4˚C)] was added to a new 10 cm plate. A plate of confluent *D. melanogaster* S2 cells were washed by gently adding 5-7mL of room temperature media and gently swirling before aspirating the media. Afterwards another 5-10mL of fresh room temperature media was added and

gently pipetted up and down to peel the cells off the bottom of the plate. 3mL of this cell suspension was added to the new plate and gently swirled to help cells attach to the bottom of the plate. The plate was incubated at 25˚C, with humidity of the incubator maintained by autoclaved Milli-Q water. Confluency of 100% is reached within 7 days but repeats of a 1:5 split maintenance is conducted at around ~80% confluency.

Cell lysis assay

For the cell lysis assay, S2 cells were cultured in a 24-well plate with 0.5 ml medium until cells reached ~75% confluency. After reaching desired confluency, Sc-sPLA$_2$ and bee venom sPLA$_2$ (from EnzChek™ Phospholipase A2 Assay Kit) were filtered with a 0.45 µm filter before being added to the cell medium. 10 µgs of Sc-sPLA$_2$ and 1 µg of bee venom sPLA$_2$ were added, and the cell medium was diluted with filtered 20 mM Tris, 300 mM NaCl, and pH 8.0 buffer to a final volume of 0.6 ml (600 µl). Cells were incubated at 25°C for 24 hours. After incubation, the supernatant was aspirated and cells were resuspended with a new volume of 600 µl filtered 20 mM Tris, 300 mM NaCl, and pH 8.0 buffer. 5 µl of cells were then added to 5 µl of trypan blue for a total of 10 µl, and then placed on a dual-chamber slide where percent of live cells were quantified by a Bio-Rad TC20 cell counter. Statistics were shown as one-way ANOVA, with error bars depicting mean with SEM.

pHrodo phagocytosis

Injections were carried out as previously described for *S. pneumoniae* except with a 4 mg/ml suspension of pHrodo Red *E. coli* BioParticles Conjugate for phagocytosis as a substitute for the bacterial solution. This solution was diluted 1:4 in PBS containing

either 5, 10, or 40 ng of Sc-sPLA$_2$ immediately prior to injection. A negative control of no protein was injected for analysis along with the 3 different protein doses. 3 flies were injected for each treatment group with a total of 3 biological replicates each. Injected flies were incubated at 28°C with 50% CO_2 for 1 hour. After incubation, the dorsal side of the abdomen of the flies was imaged with an X-Cite® 120Q fluorescence lamp, and a ZEISS Axiocom 506 Color microscope camera attached to a ZEISS SteREO Discovery V12 microscope at 10x magnification. ImageJ software was used to measure area-normalized corrected total fluorescence of isolated red channels. Statistics were shown as one-way ANOVA, with error bars depicting mean with SEM.

Hemocyte perfusionMethods were adapted from [70]. For hemocyte extraction 5–7-day old male Oregon R flies were anesthetized with CO2, washed in 70% ethanol and air dried before cutting the last abdominal segment with a clean scalpel. A fine glass capillary needle was inserted in the anterior part of the thorax and PBS was perfused under air pressure using a MINJ-FLY high-speed pneumatic injector (Tritech Research, CA). Flushed hemocytes were collected onto paraffin film. Flushed hemocytes from 10 flies were pooled together onto paraffin film and taken up with a pipette and placed into a 1.5 mL microcentrifuge tube. Pooled hemocytes were gently mixed by pipetting. 5 µl of hemocytes were added to 5 µl of trypan blue for a total of 10 µl, and then placed on a dual-chamber slide where percent of live cells were quantified by a Bio-Rad TC20 cell counter. Statistics were shown as one-way ANOVA, with error bars depicting mean with SEM.

In vitro metabolomics

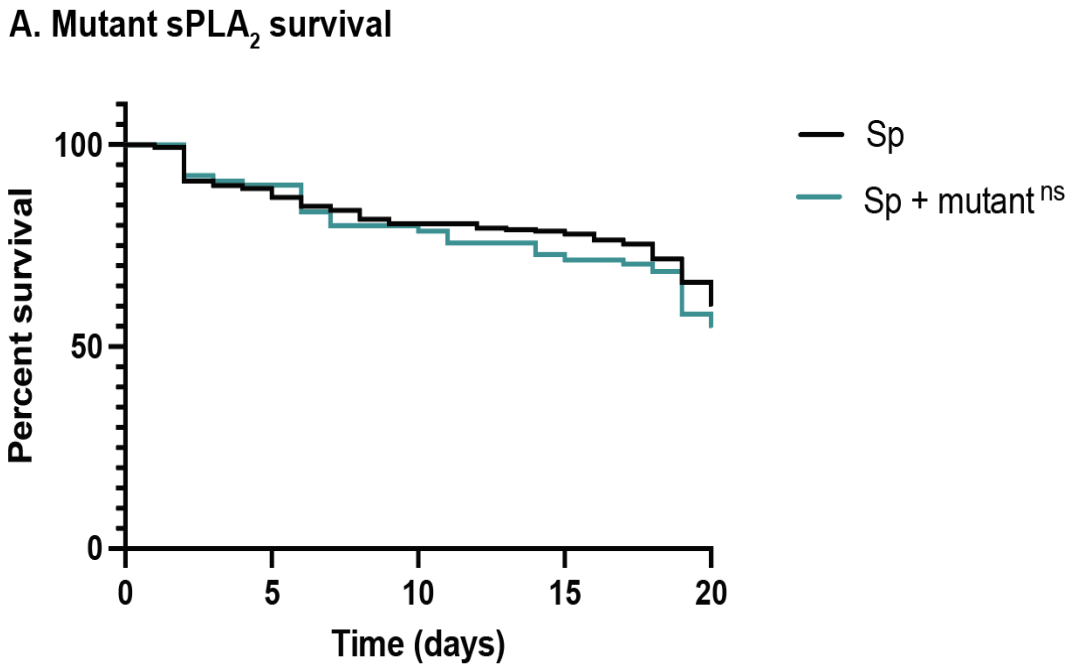
The targeted analysis was performed using a QQQ XEVO TQ-XS (Waters Corp., Milford, MA, USA) at the UC Riverside Metabolomics Core. The liquid chromatography-mass spectrometry (LC-MS) autosampler was maintained at 4°C prior to analysis. For the analysis, an injection volume of 2 µL of the extract was used. The separation was performed on the Waters XSelect CSH Phenyl-Hexyl column (3.5 µm, 3.0 × 100 mm (Waters Corp., Milford, MA, USA). The flow rate was maintained at 0.8 mL/min at 30 C. Mobile phase A consisted of ACN/water (95/5, v/v, pH=8.0) containing 25 mM AcNH4 and Mobile Phase B consisted of ACN/water (50/50, v/v, pH=7.5) containing 25 mM AcNH4. The gradient separation method was used as follows: 8 min (0–0.2 min 99% B; 0.2-3.0 min 99% B to 1%B, 3.0-3.8 min 1% B; 3.8-3.9 min 1% B to 99% B; 3.9-8.0 min 99% B. The MS data were acquired in multiple reaction monitoring (MRM) mode. The electrospray ionization was performed in positive ion mode. The source and desolvation temperatures were maintained at 150°C and 600°C, respectively. The desolvation gas was set to 1100 L/h and cone gas to 150 L/hr and the collision gas was set to 0.15 mL/min. All gases used were nitrogen, other than the collision gas which was argon. The capillary voltage was 1.5 kV. The data was normalized for relative abundance against the internal standard (LPC 17:0). Targeted data processing was performed with the open-source Skyline software [69].

Hemolymph only metabolomics – UCSD

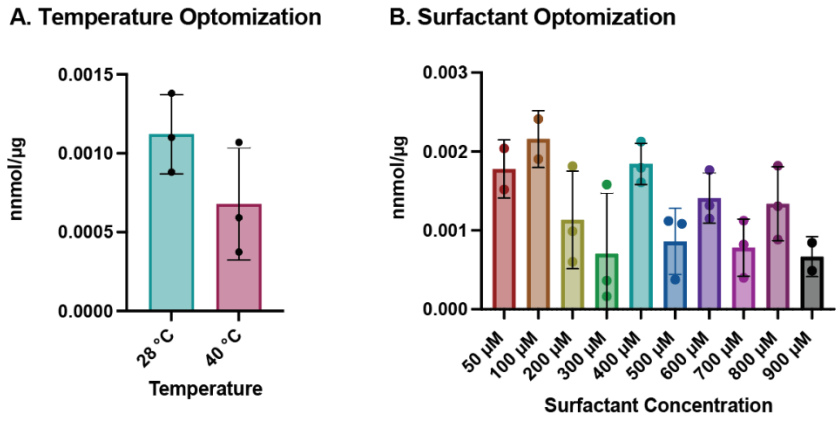
A mix of 26 deuterated internal standards was added to 10uL of hemolymph. Eicosanoids were extracted by solid phase extraction (SPE) using Phenomenex Strata-X polymeric

reversed phase columns. Samples were brought to dryness and taken up in buffer A (water/acetonitrile/acetic acid 60/40/0.02, v/v/v). Samples were analyzed using a Waters Acquity UPLC interfaced with an AB Sciex 6500 QTrap instrument. Chromatographic separation was achieved by a step gradient starting with100% buffer A to 100% buffer B (acetonitrile/isopropanol 50/50, v/v) over 5 min. Standard curves were obtained in parallel using identical conditions. Data analysis was performed with Analyst and Mulitquant software packages [71]. We monitored 159 MRMs.

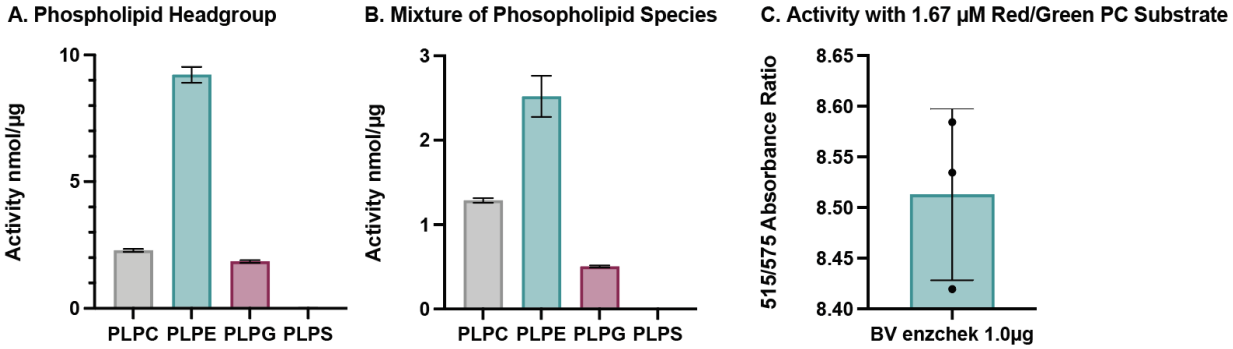
SUPPLEMENTAL INFORMATION



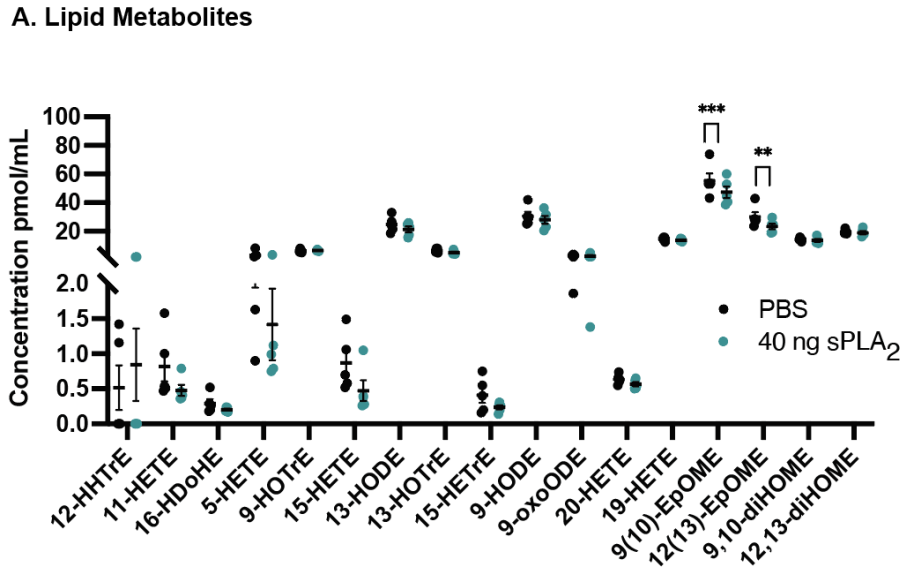
Supplemental Figure S1.1 Sc-sPLA$_2$ mutant survival assay. Inactive mutant sPLA$_2$ does not show an immunomodulatory effect. Flies were injected with 2,000 cells S.p. or 2,000 cells S.p. plus mutant sPLA$_2$ and survival was monitored for 20 days. There is no significant difference between the two treatment groups. Each treatment group represents at least 180 flies on the Kaplan Meier graph with significance shown as log-rank Mantel-Cox) test.



Supplemental Figure S1.2. Lipidomics optimization assay. A) Enzyme activity of Sc-sPLA$_2$ towards 100 µM of PLPC at two different temperatures (28°C and 40°C). B) Enzyme activity of Sc-sPLA$_2$ towards 100 µM of PLPC with varying concentrations of surfactant. Negative control values were subtracted as background from each reaction (A and B). Experiments were done in either duplicate or triplicate. All statistics shown as unpaired t-test, error bars depict mean with SEM.



Supplemental Figure S1.3. Bee venom sPLA$_2$ lipidomics assay panel. A) Enzymatic activity of Bee venom sPLA$_2$ towards 100 µM of PLPC, PLPE, PLPG and PLPS. B) Enzymatic activity of Bee venom sPLA$_2$ towards 100 µM mixture (20 µM each) of PLPC, PLPE, PLPG and PLPS. Negative control values were subtracted as background from each reaction (A and B). C) *In vitro* activity data of Bee venom sPLA$_2$ at 1.0 µg. Fluorescent emission intensity was measured at 515 and 575 nm and recorded as a ratiometric value. Negative control was subtracted as background from both absorbance values before calculating the ratio. Substrate used was a Red/Green Bodipy labeled PC. Experiments were done in triplicate. All statistics shown as unpaired t-test, error bars depict mean with SEM.



Supplemental Figure S1.4. Fly hemolymph oxylipin lipid metabolite panel. Injection of recombinant Sc-sPLA$_2$ reduces 9,(10)-EpOME and 12,(13)-EpOME in fly hemolymph 12 hours post injection. Flies were injected with PBS or 40 ng sPLA$_2$ and pooled hemolymph was analyzed for downstream lipid metabolites 12 hours post injection. 9(10)-EpOME and 12,(13)-EpOME showed a significant reduction in the 40 ng protein group. Experiments were repeated 5 times with 200 flies per treatment group. Out of 131 metabolites, 17 were detected in fly hemolymph samples. Error bars show mean + SEM with statistics shown as unpaired t-test.

STATISTICS

All statistics were done with GraphPad Prism 9.1.0 for Mac. Statistical significance indicated with asterisks indicating the following p-value cut offs: 0.05-0.033*, 0.033-0.002**, 0.002-0.0001*** and <0.0001****. Data with an n-value more than 10 was checked for normality and lognormality and the appropriate tests were performed based on these results. For data with and n-value less than 10, normal Gaussian distribution was assumed. Survival curves were analyzed by plotting one treatment group and its control to measure the curve comparison.

AUTHOR CONTRIBUTIONS

SCP carried out toxicity and immunomodulatory studies, conceptualized, planned, and carried out lipid metabolite studies, and wrote and designed the manuscript. OKO designed plasmid construction, produced and purified recombinant protein for the study, performed enzymatic and lipidomic protein activity assays, assisted in the phagocytosis experiments, and wrote and designed the manuscript. PA performed fly injection experiments for hemocyte counts and immunomodulatory studies, and contributed to the writing of the manuscript. SN, SM-B, and IC assisted in toxicity, immunomodulatory, and mass spectrometry assays, and in maintaining and scoring flies. KA assisted in lipid metabolite studies. AB helped design, optimize, and carry out the mass spectrometry activity assays. HD assisted in recombinant protein production and optimization of protein purification. AD secured funding, managed the project, and assisted in the development of the manuscript. All authors contributed to the article and approved the submitted version.

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Chapter 2

Characterizing Immunostimulatory Lipids and Endogenous PLA_2 enzymes in *Drosophila melanogaster*

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This chapter includes research that will be sent for publication in the Journal of Lipid Research, where I will be credited as a co-first author. The Heat map and CG1583 activity data in this paper will be used in a future publication delineating endogenous *D. melanogaster* endogenous $sPLA_2$ in innate immunity. While the author contributions section will provide an overview of everyone's contributions, I would like to provide a more detailed outline of my specific contributions to the overall work.

Firstly, I assisted Sophia (SCP) by counting the flies that were injected for the survival assays. I calculated and designed the dose for each lipid and prostaglandin used in the survival assays. The figure used to conceptually map out the mechanistic pathway for $sPLA_2$ mediated lipid signaling in this chapter and future publication, was generated by combining two figures Sophia and I both created. I played a role in the PO assay by recording the absorbance of the plate assay. Moreover, I was responsible for the RNA

extraction and RT-qPCR for the AMP immunoassay and performed the subsequent data analysis. In relation to the pHrodo phagocytosis assay, I assisted in visualizing and capturing photos of the flies. I also processed the data using ImageJ and conducted data analysis for the purpose of graphing. I designed the Heat Maps for the endogenous PLA_2 genes visually with GraphPad Prism, and quantitatively by utilizing the Flysick-*seq* transcriptomic source. Additionally, I took charge of designing and carrying out all protein recombinant expressions and purifications. I was responsible for designing and executing the enzymatic assays utilized for analysis via mass spectrometry. I also performed data analysis for these assays. Lastly, I wrote most of this study for future submission to the Journal of Lipid Research. The submission will undergo multiple rounds of revision by the participating authors, but as it stands my writing is the bulk of this study.

ABSTRACT

Eicosanoids are C20 polyunsaturated fatty acids (PUFAs) that play a vital role in mammalian and insect biological systems including development, reproduction, and immunity. Recent research has shown that insects have significant but lower levels of C20s in circulation in comparison to C18s. It has been previously hypothesized that eicosanoids are synthesized from C18 precursors such as linoleic acid. Here we show that *Drosophila melanogaster* displays higher levels of C18s in the hemolymph, which are depleted post bacterial infection. This research shows these depleted fatty acids as well as certain prostaglandins rescue the outcome of infection. Downstream immune readouts showed that one of these fatty acids, linoleic acid stimulates phagocytosis by hemocytes, while eliciting reduced AMP production. In totality this work identifies PUFAs that are involved in immunity and supports the notion that Drosophila utilizes immunostimulatory and immunosuppressive lipid signaling to mitigate bacterial infections. Our understanding of immune signaling in the fly and its analogies to the mammalian system will allow for an even more detailed use as a model organism in immune studies.

INTRODUCTION

Polyunsaturated fatty acids (PUFAs) play crucial roles in the development, regulation of bodily functions, and immunity across different organisms. In mammals, PUFAs serve as substrates for three major lipid biosynthesis pathways, leading to the formation of eicosanoids. Upon hydrolysis from the membrane by phospholipase A_2 (PLA_2), eicosanoid precursors such as arachidonic acid (AA) eicosapentaenoic acid [1] and

docosahexaenoic acid (DHA) can be converted into prostaglandins (PGs) by cyclooxygenases (COXs), leukotrienes and hydroxyeicosatrienoic acids by lipoxygenases (LOXs), and epoxyeicosatrienoic acid (EET) by cytochrome P450 (CYP)[2-6]. In insects it has been suggested that endogenous PLA_2s cleave linoleic acid (LA) which can be converted to AA by desaturases and long chain fatty acid elongase [7, 8]. While previous studies suggested that insects lacked C20 PUFAs, new research showed that were able to be detected in significant concentrations in *Drosophila melanogaster* [9, 10]. Although the overall research on eicosanoids in insects is relatively limited, it has been demonstrated that they play significant biological roles. For instance, prostaglandins have been found to affect the egg-laying behavior of crickets, such as *Teleogryllus commodus*, influencing their reproduction [11, 12]. In silkmoths, inhibition of PG synthesis interferes with follicle development, and products of LOX and COX in *Rhodnius plexus*' ovaries regulate the uptake of *Rhodnius* heme binding protein [13-15]. Similarly, in ticks, PGE2 influences fluid secretion rates and composition, with inhibition of PLA2 and COX resulting in decreased fluid secretion and stimulation of salivary glands with PGE2 leading to various physiological responses [12, 13].

In humans, eicosanoids are well-known as lipid mediators involved in immune responses. Interestingly, research suggests that eicosanoids also participate in cellular and humoral immune responses in insects. The role of eicosanoids in insect immunity was first discovered in *Manduca sexta*, where inhibition of PLA_2 decreased the organism's ability to clear bacteria from the hemolymph, a phenotype rescued by the addition of arachidonic acid [14]. Further investigations on insect immune responses revealed that cellular

immune markers, such as nodulation and melanotic encapsulation, were influenced by eicosanoids [15-17]. In addition, PUFAs also demonstrate bactericidal effects directly on gram positive bacteria [18, 19]

Drosophila melanogaster, commonly known as fruit flies, serve as a valuable model organism for studying disease. Fruit flies possess physical barriers and innate immunity, including cellular and humoral components. Cellular immunity involves phagocytosis and encapsulation, while humoral immunity leads to the production of antimicrobial peptides (AMPs) [20, 21]. AMP production is regulated by two signaling pathways: the Toll pathway and the immune deficiency (imd) pathway, which bear similarities to mammalian Toll-like receptor/interleukin 1 receptor signaling cascade and TNF-R pathway, respectively [20]. The connection between PLA2-generated fatty acids and the LPS-activated imd pathway has been established, with suppression of the imd pathway observed when PLA2 inhibitors are used and rescued by the addition of arachidonic acid [22]. Initially, the absence of COX gene homologs typically found in mammals within the genome of D. melanogaster raised intriguing questions about the synthesis of eicosanoids. However, this mystery was unraveled with the discovery of a unique COX-like peroxidase called peroxinectin (Pxt), which presents a novel mechanism for prostaglandin (PG) synthesis in fruit flies and insects [23].

Despite considerable research focusing on lipids in the context of insect immunity, the precise role of eicosanoids and other lipid molecules in the immune responses of D. melanogaster has not been sufficiently characterized. Given previous knowledge, a model hypothesizing the pathway for eicosanoid production in insects was designed (Figure 1)

This study presents strong evidence elucidating that PUFAs cleaved by endogenous PLA_2s display immunomodulation in *D. melanogaster*.

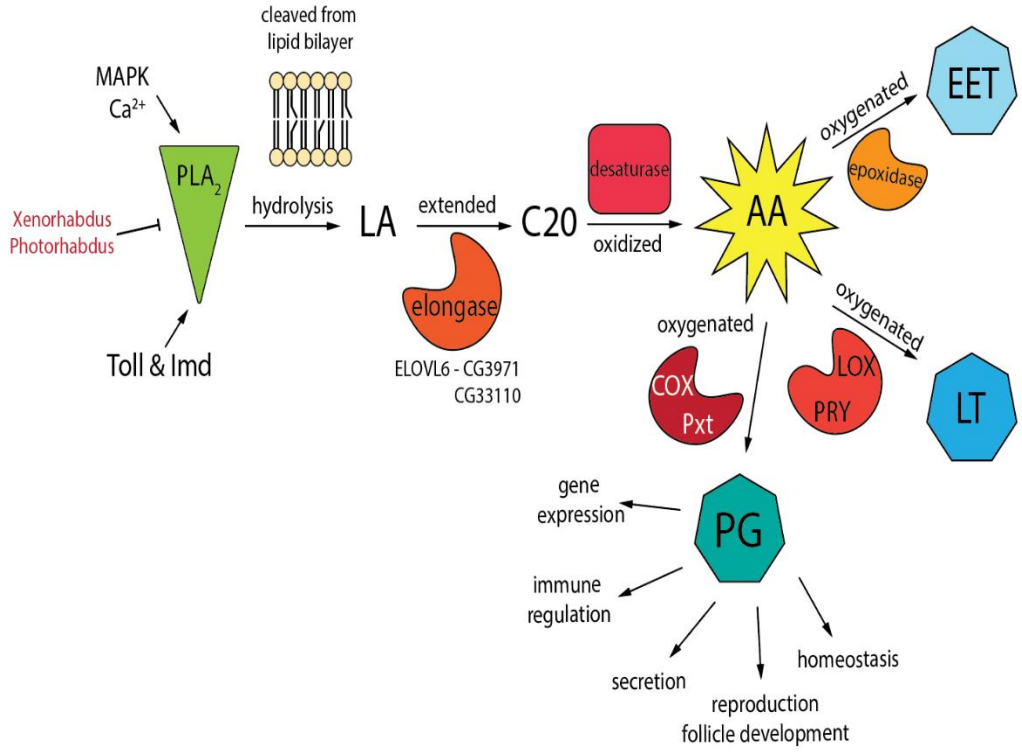


Figure 2.1 Proposed lipid signaling pathway in insects. A diagram of the lipid signaling pathway potentially involved in insect immunity.

RESULTS

Lipid depletion in fly hemolymph during bacterial infection.

In order to investigate the role of lipids in immune signaling in the model organism *Drosophila melanogaster*, we conducted a study using *Streptococcus pneumoniae* (*S.p.*) as an immune challenge. The objective was to identify changes in lipid metabolites following infection [24]. Our initial focus was on lipids present in the hemolymph, which

are released from the cell membrane through the action of a phospholipase A2 enzyme. We analyzed the hemolymph samples of 5-7 day old male flies which comprised of 2,000 individuals injected with either PBS or *S.p.*, and were able to detect 13 out of 86 targeted lipid metabolites. Interestingly, all the lipids detected in this study belonged to the C18 class, although previous studies have identified C20 lipids in fly hemolymph [9, 24]. Among the 13 lipids detected, 6 exhibited a significant decrease 12 hours after infection. Some of these lipids were upstream precursors like oleic acid (OA), LA and alpha linolenic acid (ALA). While others, such as 9-oxo-ODE, 13-oxo-ODE, and 9-oxo-OTrE, represented oxidized metabolites located further downstream oxylipin pathway (Figure 2) [25, 26]. Similar effects were observed at 6 hours post infection, with the depletion of the downstream oxidized derivatives 13-oxo-ODE, and 9-oxo-OTrE (Supplemental figure 1), indicating that lipid metabolites undergo alterations even within a short period after infection.

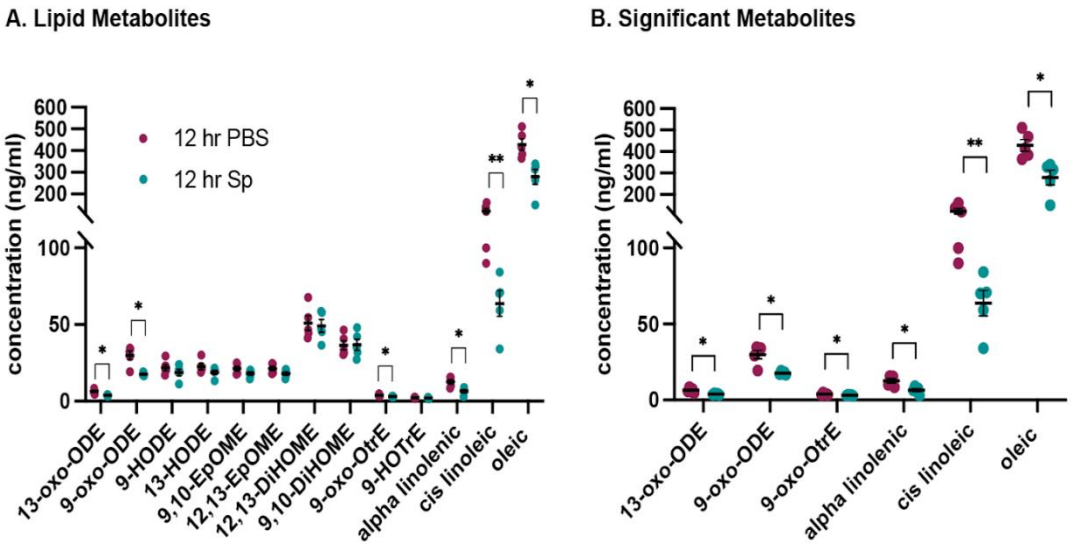


Figure 2.2 Fly hemolymph lipid metabolite panel 12 hours post injection. A) The lipid metabolites in fly hemolymph are significantly reduced 12 hours after a *S.p.*-induced immune challenge. Flies were injected with 7,000 *S.p.* cells, and hemolymph samples were collected for mass spectrometry analysis at the 12-hour mark post-injection. B) Several lipid metabolites, including 13-oxo-ODE, 9-oxo-ODE, 9-oxo-OtrE, α-linolenic acid, cis linoleic acid, and oleic acid, showed significant reductions in infected flies. The experiments were repeated five times, with each treatment group consisting of 200 flies per replicate, totaling 2,000 flies. The error bars represent the mean + SEM, and statistical analysis was performed using Welch's t-test.

PUFAs, eicosanoids and oxylipins increase survival during infection.

To investigate the impact of specific lipids on the course of infection, we co-injected the lipids that showed significant reductions post-infection, along with arachidonic acid and prostaglandins, with *S. pneumoniae*. We were able to observe dose-response survival curves upon treatment with the lipids. Some upstream lipids, such as ALA and OA, demonstrated positive effects on flies at doses as low as 50 and 100 µM (Figure 3A and 3D), while LA required a higher dose of at least 250 µM to elicit a similar response (Figure 3B). AA, a key eicosanoid and prostaglandin precursor that is found at respectable levels in fly hemolymph, was included in our rescue study and even a 50 µM

dose offered significant protection against death caused by bacterial infection [9](Figure 3C). Strikingly, AA, LA, ALA, and OA were able to completely rescue flies from bacterial infection at higher doses of 500 μM and 1 mM, increasing the 15-day survival rate from one percent in bacteria-only injected flies to over 90 percent in co-injected flies (Figure 3A, B, C, D). Not all lipids exhibited this protective effect, as the geometric isomer of linoleic acid, linoelaidic acid, showed no protective effect even at the highest tested dose of 1 mM (Supplemental Figure 2). This demonstrates the specificity of the protective effects of these lipids, suggesting their potential involvement in immune signaling in flies. Lastly, we observed an increase in survival after treatment with prostaglandins (Figure 4). The prostaglandins were not detected in our fly hemolymph samples but have been proposed to be a product of putative eicosanoid biosynthesis pathway in the fly [27]. Prostaglandin F2α and F2β both enhanced survival in flies at doses of 100 and 250 μM (Figure 4A). Prostaglandin D2 exhibited a rescue effect only at the 250 μM dose, while Prostaglandin E2 displayed this effect at doses of 50, 100, and 250 μM (Figure 4B).

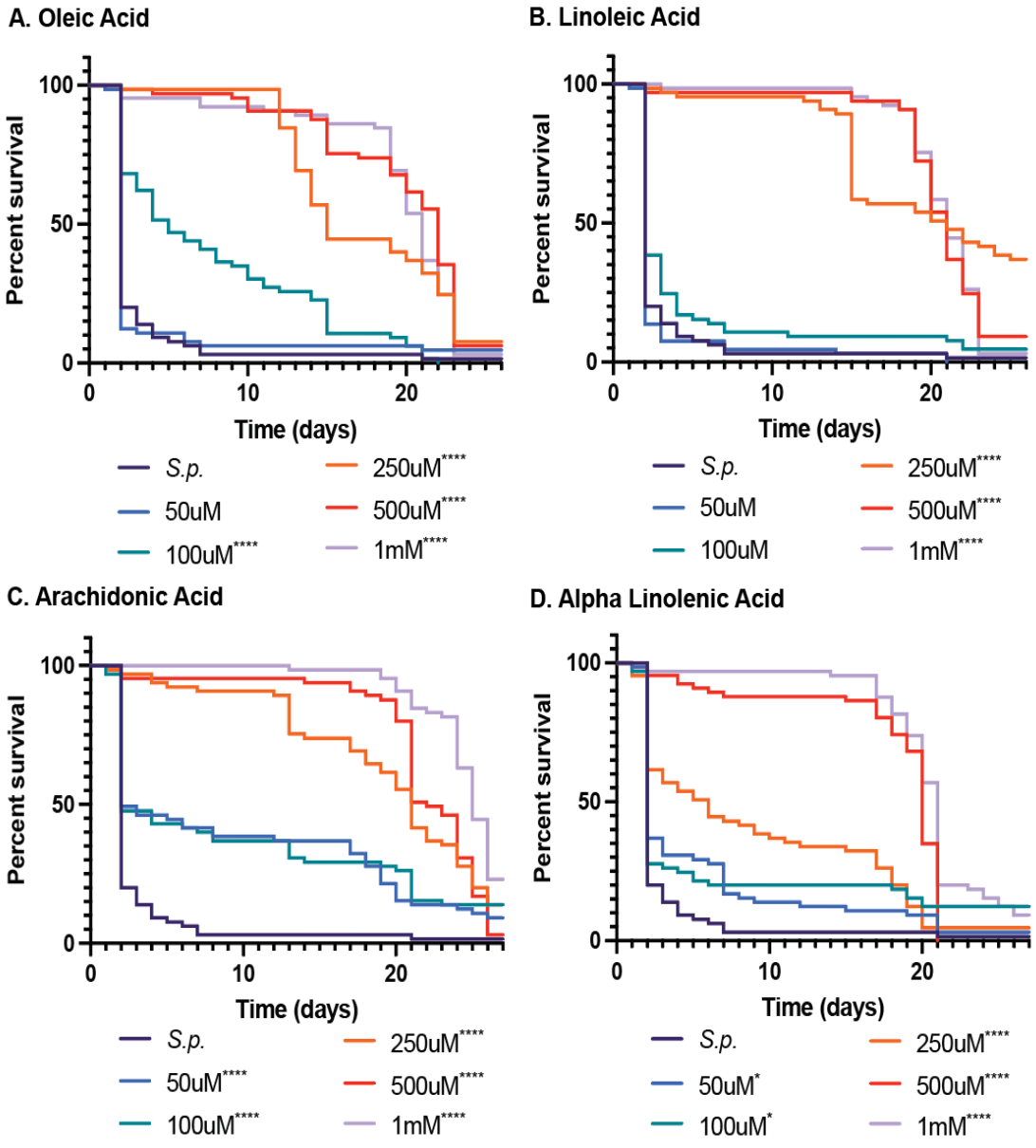


Figure 2.3 Fatty acid survival assay. In a dose-dependent manner, both upstream lipids and downstream oxylipins demonstrate the ability to rescue bacterial infections. Flies were injected with 7,000 *S.p.* cells along with various lipid doses. A) OA exhibits a significant beneficial effect on infection outcomes starting at 100 mM. B) LA shows beneficial effects at 250 mM. C&D) AA and ALA are effective even at the lowest dose of 50 mM. The dark blue bars represent the *S.p.*-only injected controls. The experiments were replicated at least three times, with a total of at least 180 flies per treatment group. Statistical analysis was performed using the Log-rank test, and asterisks are used to indicate significant differences next to the experimental group labels below the graph.

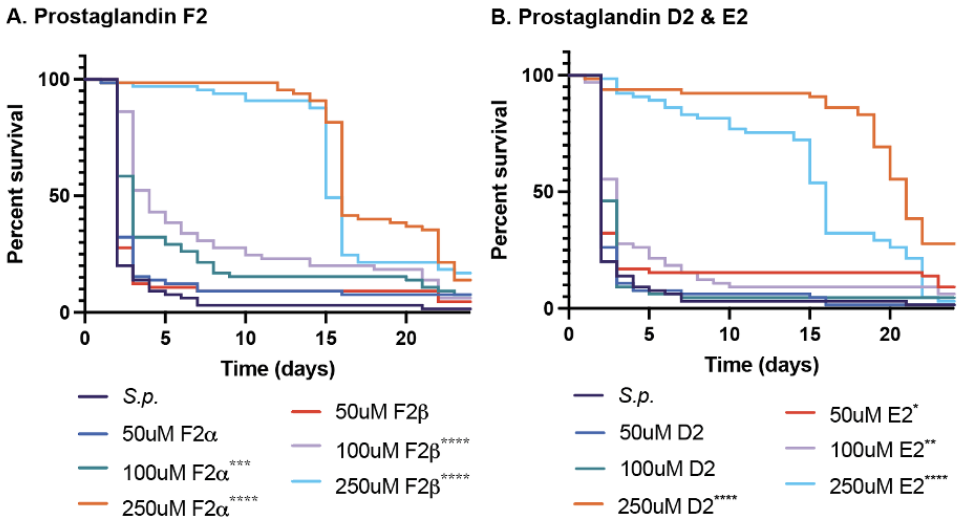


Figure 2.4 Prostaglandin survival assay. Downstream prostaglandins have a significant positive impact on the outcome of bacterial infections. Flies were injected with 7,000 *S.p.* cells along with varying doses of prostaglandins. A) Prostaglandins $F2_{\alpha}$ and $F2_{\beta}$ demonstrate substantial improvement in infection outcomes at doses of 100 and 250 mM. B) Prostaglandin D2 exhibits beneficial effects only at the highest dose of 250 mM, while prostaglandin E2 shows a positive effect starting at a lower dose of 50 mM. The experiments were replicated at least three times, involving a minimum of 180 flies per treatment group. Statistical analysis was performed using the Log-rank test, and asterisks are used to indicate significant differences next to the experimental group labels below the graph.

LA suppresses Toll and Imd pathway but stimulates phagocytosis along with AA and PGE_2.

In order to delineate how PUFAs stimulate immunity, we assessed various indicators of immune response, such as PO activity, AMP production, and phagocytosis. Phagocytosis is a crucial cellular immune process in insects and was visualized and quantified in *D. melanogaster* through the injection of fluorescently labeled conjugates of E. coli [32, 33]. These conjugates fluoresce upon exposure to the lysosome's low pH environment. We performed coinjections of these conjugates with a 250µM concentration of LA, AA, and PGE2 to examine any changes in phagocytosis. Our findings demonstrated that LA and PGE2 both significantly increased phagocytosis activity one hour post injection (Figures 5A, B). While AA did not display statistical significance for increase in phagocytosis,

there was still a noticeable trend for higher phagocytosis after treatment with AA (Figure 5B). Flies treated with LA exhibited an average CTF of approximately $8.3106*10^6$, while the negative control had a CTF of around $2.9106*10^6$ (Figure 5A). Flies treated with AA displayed an average CTF of approximately $7.1103*10^3$, and those treated with PGE2 showed an average CTF of approximately $8.2103*10^3$, compared to a CTF of approximately $4.6*10^3$ in the negative control (Figure 5B). Disparity in CTF magnitude between the two figures can be attributed to background lighting intensity during imaging (images were taken at separate times hence the two sets of negative controls). PO activity plays a crucial role in melanization, and we conducted a coinjection experiment using a 250 μM concentration of LA and *Listeria monocytogenes.* We used *L. monocytogenes* in order to observe any alterations in PO activity as it is a bacterium known to trigger robust melanization [28, 29]. Our results showed that treatment with LA did not lead to significant changes in PO activity compared to the Listeria-only group (Figure 5C). To further evaluate specific immune responses, we measured AMP production 24 hours after injection. The lipid treatment resulted in a significant decrease in the expression of *defensin* (Imd), *metchnikowin* (Imd), *diptericin* (Toll), and *drosomycin* (Toll), indicating a suppressive effect on the Toll and Imd pathways (Figure 5D) [30, 31].

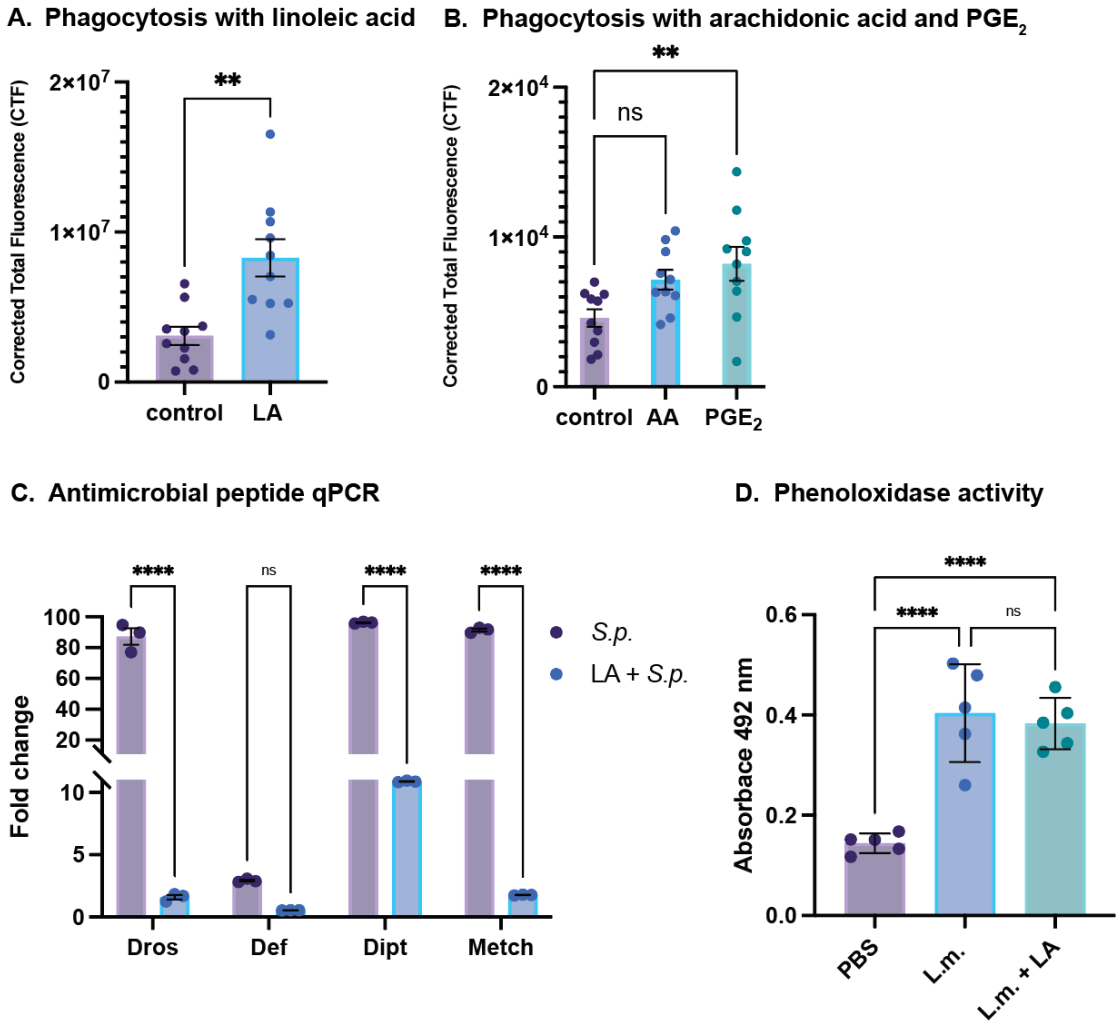


Figure 2.5 Downstream immune readouts. Specific downstream immune responses are affected by LA, AA and PGE_2. A) Phagocytic activity was measured with the pHrodo assay showing fluorescence once phagocytosed. pHrodo only injected flies show higher amounts of fluorescence. Fluorescence is increased in flies injected with 250 µM LA. B) Fluorescence is increased in flies injected with 250 µM PGE_2. AA displayed higher phagocytosis by increased average CTF, but the value was not statistically significant. Statistics displayed as ordinary one-way ANOVA with Dunnett's multiple comparisons test. Experiments were repeated 3 times with at least 3 flies per group. C) Phenoloxidase activity was measured 6 hours after injection with either PBS control, 10,000 cells *Listeria monocytogenes*, a known melanizer, or *L.m.* plus 250 µM LA. An increase in PO activity was observed in the bacteria injected group but was not altered by the presence of the lipid. Experiments were completed 5 times with 30 flies in each treatment group. Statistics displayed as ordinary one-way ANOVA with Dunnett's multiple comparisons test. Error bars depict mean with SEM. (B) Antimicrobial peptide production was measured by quantitative PCR 24 hours after injection with *S.p.* or *S.p.* plus 250 µM LA. Four different AMPs were measured, *Drosomycin* (Toll), *Defensin* (Imd), *Diptericin* (Imd), and *Metchnikowin* (Toll) were all decreased after protein injection. Statistics shown as 2-way ANOVA with a Tukey multiple comparisons test. Experiments repeated 3 times with 15 flies in each group.

***D. melanogaster* endogenous PLA_2, desaturase, and elongase genes are upregulated during infection.**

To determine which upstream lipid signaling enzymes are upregulated during infection a heat map was constructed for predicted endogenous $sPLA_2$, desaturase, and elongase genes (Figure 6). The data for the heat map was acquired utilizing a transcriptomic database generated in a study that looked at *D. melanogaster* gene regulation during a variety of infections [34]. The heat map illustrates variations in gene expression between an uncultured fly sample, quantified as the logarithm of the difference in transcripts per million (tpm). The bacterial infections and their corresponding time points in hours are presented from left to right as follows: M. luteus (12, 36, 132), E. coli (12, 36, 132), S. marcescens Type (12, 36, 132), Ecc15 (12, 36, 132), P. rettgeri (12, 36, 132), E. faecalis (12, 36, 132), S. aureus (12), P. sneebia (12), S. marcescens Db11 (12), and P. entomophila (12). The PLA2 heat map displays six sPLA2 genes (CG14507, 3009, 42237, 1583, 11124, and 17035) and three iPLA2 genes (CG6718, 7365, and 11029) (Figure 6A). The second heat map showcases two desaturase genes (CG17928 and CG13279) and two elongase genes (CG32072 and 11801) (Figure 6B).

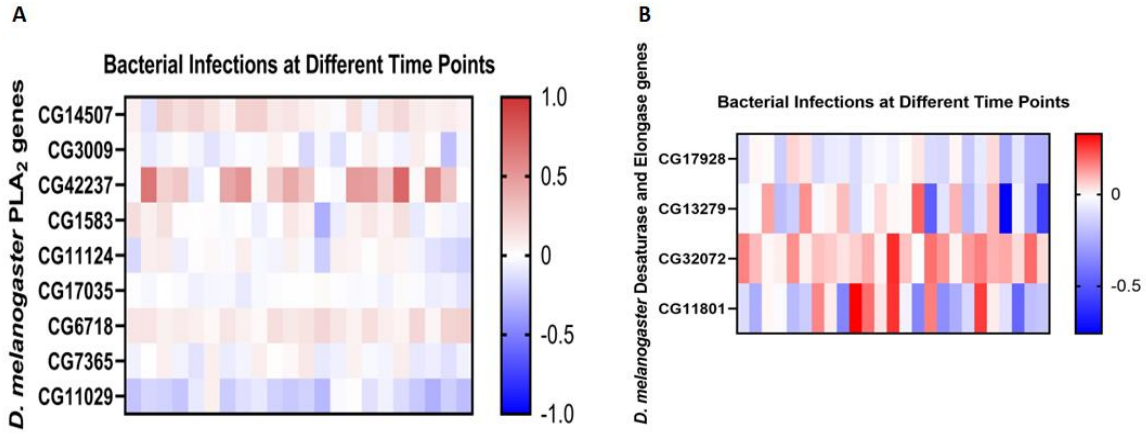


Figure 2.6 Fly enzyme heat maps. Heat map highlights the difference in gene expression from an uncultured fly sample which is quantified as the log value of the difference in transcripts per million (tpm). A) 6 out of 7 endogenous fly $sPLA_2$ enzymes were assessed due to availability of gene information in the Flysick database. B) Top 2 genes encode desaturase enzymes, and bottom two genes encode elongase enzymes. Expression measured as the log value of the difference in transcripts per million (tpm). From left to right bacterial infections and their time points in hours are reported accordingly: *M. luteus* (12, 36, 132), *E. coli* (12, 36, 132), *S. marcescens* Type (12, 36, 132), *Ecc15* (12, 36, 132), *P. rettgeri* (12, 36, 132), *E. faecalis* (12, 36, 132), *S. aureus.* (12), *P. sneebia* (12), *S. marcescens* Db11 (12), and *P. entomophila* (12).

CG1583 displays exponentially higher activity with PLPE, OA, and AA.

Lipidomics through a high-throughput mass spectrometric assay to gain a better understanding of the impact of CG1583 on lipid metabolism and its preferred targets among phospholipids [35]. This assay allowed us to investigate the in vitro activity of CG1583 on natural and synthetic membrane phospholipids in mixed micelles. The preference of CG1583 for phospholipid head groups was elucidated via utilization of four major phospholipid head groups—phosphoethanolamine (PE), phosphoserine (PS), phosphoglycerol (PG), and phosphocholine (PC)—at the *sn-3* position. The *sn-1* position consisted of palmitic acid since it can be produced de novo in Drosophila [36]. Previous studies have shown that the *sn-1* fatty acid does not affect PLA_2 activity, so no optimization was conducted for that position [35]. In terms of optimization and head group studies, LA was incorporated at the *sn-2* position, considering it was determined to

be the most prevalent PUFA in *D. melanogaster* [36]. Reactions were carried out for 30 minutes, as previous studies indicated it to be the optimal time for multiple PLA_2s [35]. The surfactant concentration was determined based on a previous study, and the enzyme concentration for the assays was determined by testing two different amounts (Supplemental Figure 3).

To determine Sc-sPLA2's preference for phospholipid head groups, assays were conducted with the lipid substrate 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphox, where "x" represents one of the four major lipid head groups (PLPE, PLPS, PLPG, and PLPC). The experiment revealed that CG1583 exhibited significantly higher activity towards PLPE compared to other head groups, with PG being the second highest (Figures 7A, B). It is worth noting that the abundance of PE has been linked to Toll pathway expression in D. melanogaster [37]. Subsequent experiments were conducted using lipid substrates with the PE head group to determine CG1583's fatty acid preference at the sn-2 position. The fatty acids used were OA, LA, and AA. OA, being an 18-carbon fatty acid with high abundance in D. melanogaster and the ability to convert to LA, was selected [24, 38]. LA and AA are precursors to immunomodulating eicosanoids, with AA being the more common precursor in mammals [5, 39, 40]. The results demonstrated that CG1583 displayed higher activity towards both OA and AA compared to LA (Figure 7C). In summary, these findings indicate that Sc-sPLA2 exhibits strong activity towards specific lipid species that have downstream effects on immunity.

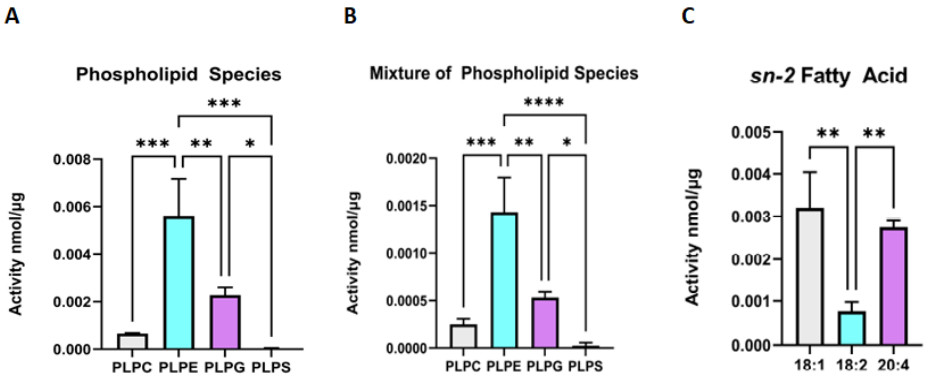


Figure 2.7 CG1583 lipidomics panel. Enzymatic activity assays revealed that CG1583 exhibited a preference for PLPE, with PLPG being the second most preferred substrate. A) The enzymatic activity of CG1583 was tested against 100 µM of PLPC, PLPE, PLPG, and PLPS. Statistical analysis was performed using an ordinary one-way ANOVA with Tukey's multiple comparisons test. Significant differences were observed between PLPC and PLPE, PLPE and PLPG, PLPG and PLPS, and PLPE and PLPS. B) The enzymatic activity of CG1583 was assessed using a mixture of 100 µM (20 µM each) of PLPC, PLPE, PLPG, and PLPS ($p <$. Statistical analysis was conducted using an ordinary one-way ANOVA with Tukey's multiple comparisons test. Significant differences were observed between PLPC and PLPE, PLPE and PLPG, PLPG and PLPS, and PLPE and PLPS. C) The enzymatic activity of CG1583 was measured against 100 µM of PLPE species with different sn-2 fatty acids: OA (18:1), LA (18:2), and AA (20:4). Statistical analysis was performed using an ordinary one-way ANOVA with Tukey's multiple comparisons test. Significant differences were observed between 18:2 and 20:4, as well as between 18:1 and 18:2. Negative control values were subtracted from each reaction.

DISCUSSION

The innate immune response in *Drosophila* involves two pathways: the humoral immune response, which produces antimicrobial peptides, and the cellular immune response, which involves hemocyte cells controlling pathogen growth in circulation [20]. The immune response mechanisms in Drosophila and mammals are highly conserved [21]. Previous research indicates that bacterial infections increase phospholipid synthesis in the fat body, the main organ for humoral immune response in Drosophila, which can be converted to eicosanoids [37].

We demonstrate that the lipids depleted during infection can rescue the infection even at low doses. This suggests that providing more eicosanoid precursors to flies enables the

production of more pro-immune signaling molecules, resulting in a beneficial effect on infection outcomes. Notably, the ability to rescue the infection is specific to lipids feeding into the eicosanoid pathway and being converted by specific enzymes, as the geometric isomer of linoleic acid, linoelaidic acid, does not have the same rescuing effect.

Prostaglandins, synthesized from arachidonic acid by peroxinectin in Drosophila, play critical roles in development, reproduction, and immunity [23]. The ability of prostaglandins, particularly PGE2, to rescue the infection at low doses supports the hypothesis that Drosophila and other insects can synthesize downstream eicosanoids from C18 derivatives. The hypothesis that C18 lipids are converted to downstream immunomodulatory C20 eicosanoids is further strengthened by the ability of C18s LA, ALA, and OA to rescue immunity along with AA (Figure 3). It is important to note the PUFAs used in this study are also bactericidal to gram-positive bacteria, and thus when attempting to plate *S.p.* coinjections for CFUs we were not able to see any growth (data not shown)[18, 19]. When plating for CFUs the geometric isomer of linoleic acid, linoelaidic acid, that displayed no rescue effect in the survival assays was also able to inhibit CFU growth. It is likely that while the structural components that make these PUFAs bactericidal to gram positive bacteria, can affect CFU plating, they did not interfere with the survival assay coinjections. Coinjections were done swiftly upon mixture of the bacteria and lipids (in less than 5 minutes) and once in the hemolymph the mixture circulated, indicating the phenotype displayed comes from other mechanisms affected by the PUFAs. Prostaglandins F_2, D_2 and E_2 are all able to rescue the infection

with E_2 having the most beneficial effect Prostaglandins F2, D2, and E2 all can rescue the infection, with E2 having the most beneficial effect. Interestingly, PGE2 has a proposed direct synthesis pathway in insects [41]. Eicosanoid receptors are likely highly conserved, and the ligands capable of activating downstream immune pathways are diverse, although only a few are endogenously found in flies.

We evaluated the effects of LA, the hypothesized C20 precursor in insects, on downstream immunity in the fly. Fly immunity starts with pathogen specific recognition by the toll and imd pathways, which then leads to either a cellular immune response by specialized hemocytes, or a humoral immune response *via* production of Toll- or Imd-specific AMPs secreted from the fat body [19, 26]. Melanization is independent of the Toll and Imd pathways and is dependent on the proPO-PO cascade [22, 23]. Our findings showed that LA had no effect on PO activity (Figure 5C) but caused a reduction in the expression of the AMPs *Metchnikowin, Diptericin, Defensin* and *Drosomycin* (Figure 5D), while simultaneously significantly increasing phagocytosis (Figure 5A). PGE_2 also increased phagocytosis, and AA displayed an increase in average CTF values even though it was not statistically significant, indicating AA increases phagocytosis as a trend (Figure 5B). We hypothesize that this was due to the time point for observing fluorescence of the assay being too early for AA to generate a significant difference. Methods for the pHrodo Red *E. coli* BioParticles conjugate state that fluorescence can be observed after 30-60 minutes, with many experiments opting to observe fluorescence generally after 2 hours. A longer time point of 2 hours potentially could have established a significant difference for AA. The ability for LA, PGE_2, and to a statistically non-

significant degree, AA, to stimulate phagocytosis provides molecular mechanistic explanation for increased survival of the fly during the survival assays. *E. coli* is gram negative and thus the PUFAs would not have been able to be bactericidal to the pathogen during the coinjection process. This gives credence to the PUFAs having both a bactericidal and immunostimulatory effect in the fly. No change in PO activity from coinjected *L.m* plus lipid, in comparison to *L.m.* only treatment group, also validates the coinjections were unaltered by the bactericidal properties of the PUFAs. *L.m.* is gram positive like *S.p.*, and thus any bactericidal effects on the *L.m.* dose would have been observed by a reduction in PO measurement. As for the AMP reduction, it is possible that with increased phagocytosis of the invading pathogen, there is less Spätzle binding (Toll) and LPS detection (Imd) which leads to less activation of the Toll and Imd pathway. There is also the potential of AMP production operating as a negative feedback loop to increased cellular immune response by the PUFAs, preventing a hyperactive immune response to increase tolerance. Overall, these findings show that LA suppresses both the Toll and Imd pathways, while stimulating the cellular immune responses through increase of phagocytosis.

Six of seven $sPLA_2$ genes, three $iPLA_2$ genes, and two elongase and desaturase genes were examined for expression profile during infection. Analysis was done by utilizing a Flysick-*seq* transcriptomic database that assessed gene expression of *Drosophila melanogaster* during various time points with a variety of bacterial pathogens. Upregulation and downregulation were assessed in the heatmap relative to the grouping of the expression values. For the PLA_2 heat map, $sPLA_2$ CG42237 had the highest

expression followed by iPLA$_2$ gene CG6718, then sPLA$_2$ genes CG14507 and CG1583. Future experimentation with CG42237 would provide useful insight on whether an endogenous immune mediating lipid signaling pathway can be triggered. For the desaturase and elongase heat map, the two elongase genes CG32072 and CG11801 had highest expression, followed by the two desaturase genes CG13279 and CG17928. This result implies that extension of C18 to C20s is a more upregulated aspect of a potential lipid signaling pathway than oxidation reactions during infection. Future experimentation will be needed to validate this observation, and further evaluate whether C18 to C20 conversion is increased during infection.

We employed a lipidomics approach using a high-throughput assay based on mass spectrometry to investigate the lipid preferences of CG1583 in vitro. This data would shed light on the endogenous lipid targets of CG1583 in *D. melanogaster*. Our findings revealed that CG1583 exhibited significantly higher activity with phosphatidylethanolamine (PE) as a substrate compared to other lipid headgroups. This is noteworthy because PE is the most abundant phospholipid in cellular membranes of *D. melanogaster* [37, 42]. The substantial catalytic activity of CG1583 towards PE lipids suggests a potential advantage for efficiency in cleaving PUFAs that can stimulate immune responses. Additionally, we utilized the high-throughput assay to determine CG1583's preference for fatty acid side chains at the *sn-2* position. The in vitro assay demonstrated that CG1583 exhibited the highest activity with oleic acid (OA) and arachidonic acid (AA) at the *sn-2* position. Although activity against linoleic acid (LA) was detectable, it was noticeably lower compared to OA and AA. This finding is

intriguing as it further supports the influence of $sPLA_2$ structure and physical properties on the activity profile [9, 35]. CG1583's significant activity with OA is strategic, as it can be converted into the eicosanoid precursor LA [38]. LA, being the most abundant polyunsaturated fatty acid in insects like D. melanogaster, along with AA, serves as a precursor for generating eicosanoids involved in downstream immune responses [36, 39]. The higher activity levels exhibited by CG1583 against these two PUFAs indicate its potential to stimulate immunity by generating proinflammatory eicosanoids.

In mammals, the eicosanoid pathway from arachidonic acid branches into prostaglandins, leukotrienes, and epoxyeicosatrienoic acids, which communicate proinflammatory or anti-inflammatory signals [39]. This study sheds light on a potential conserved system in flies where lipid signaling may also induce pro- or anti-immune responses, depending on the activated pathways. Overall, our findings provide further evidence of a functional eicosanoid biosynthesis pathway in fruit flies and the involvement of eicosanoid lipid signaling in innate immunity.

METHODS

Fly stock/ maintenance

Fly strains were cultivated on D2 glucose medium obtained from Archon Scientific in Durham, North Carolina. The flies were maintained at a temperature of 25°C with 50% humidity, following a 12-hour light and 12-hour dark cycle.

Bacterial stock maintenance

The methods used in this study were adapted from previous research [24]. *Streptococcus pneumoniae* was cultured by shaking in glass vials containing 5 mL of tryptic soy (TS) broth (Difco TS broth, catalase, streptomycin) at a temperature of 37°C with 5% CO2 overnight. The overgrown culture was then diluted in catalase (100 µL) and TS broth to obtain a final volume of 20 mL in a flask. The flask was incubated while shaking until the OD600 reached approximately 0.4, which took about 1 hour. Next, the culture was further diluted to a final volume of 50 mL, supplemented with 150 µL catalase, and incubated until the OD600 reached approximately 0.2 - 0.4. An OD600 value above 0.5 indicated that the culture was no longer in the logarithmic growth phase. To preserve the culture, 5% glycerol was added, and the final culture was stored in 1 mL aliquots at -80°C. For experimental use, one aliquot was thawed, followed by centrifugation at 14,000 rpm for 5 minutes. The supernatant was removed, and the pellet was resuspended in the desired amount of PBS (typically 50 - 60 µL), yielding approximately 100,000 colony-forming units (CFUs). Serial dilutions were performed to obtain the desired CFU doses. When necessary, S. pneumoniae was plated on TSA agar plates supplemented with 50 mL/L sheep's blood.

Fly lipid coinjections and survival assay.

The methods employed in this study were adapted from previous research [24]. Male flies aged 5-7 days were used for injections and metabolomics assays. The flies were anesthetized using CO2 and injected with various colony-forming unit (CFU) doses of *S. pneumoniae*. The injections were performed with precise control, delivering a total

volume of 50 nL, using a MINJ-FLY high-speed pneumatic injector (Tritech Research, CA) and individually pulled glass needles calibrated for accuracy. To examine the effects of fatty acids, different lipids including linoleic acid, arachidonic acid, alpha-linolenic acid, oleic acid, and all prostaglandins (obtained from Caymen Chemical, Ann Arbor, USA) were dissolved in ethanol. Prior to injection, the lipids were freshly diluted in phosphate-buffered saline (PBS) for co-injection purposes. Injection sites were targeted close to the junction of the abdomen and thorax, slightly ventral from the dorsal-ventral cuticle axis, which could be easily observed beneath the haltere. For survival studies, flies were injected with the CFU dose or a control of PBS and then placed in vials. Each experimental or control group consisted of 60 flies, organized in groups of 30. Flies injected with the human pathogen S. pneumoniae were maintained at a temperature of 28°C with 50% humidity. Daily monitoring of fly mortality was conducted, and the number of deceased flies was recorded. Kaplan-Meier survival curves were generated using GraphPad Prism software, and statistical analysis was performed using log-rank analysis (Mantel-Cox). Survival experiments were at least triplicated.

Phenoloxidase activity

Methods adapted from [24]. Flies were injected with 10,000 CFUs of *L. monocytogenes* to elicit an immune induced melanization cascade. Phenoloxidase activity was measured as previously described [24]. To collect hemolymph, 20-30 flies 6 hours post injection (p.i.) were pricked through the thorax and placed in a pierced 0.5 µL Eppendorf tube and covered with glass beads, then placed inside a 1.5 µL Eppendorf tube containing 30 µL of PBS. Samples were centrifuged at 10,000 g for 20 minutes at 4°C.

Using a clear 96-well plate, each well contained 160 µL L-Dopa (3 mg/mL) dissolved in phosphate buffer (37.5% 1 M potassium phosphate, 62.5% 1 M sodium phosphate, pH 6.5), 35 µL of hemolymph sample and 5 µL $CaCl_2$ (20 mM). PO activity was measured by kinetic reads at 29°C at 492 nm every minute for 120 min with 5 seconds of shaking between reads. The OD of a blank control was subtracted from all biological values. Experiments were replicated five times with three technical replicates per experiment. Data were plotted as mean+SEM by taking the peak OD value (timepoint ~ 60 min). Statistics shown as an unpaired t-tests done in GraphPad Prism.

Antimicrobial peptide gene expression - qPCR

Methods adapted from [24]. Total RNA was extracted from 15 *S. pneumonia* or *S.p.* plus 250 µM LA injected flies 24 hours post-injection using Trizol reagent (Molecular Research Center, Inc; Cincinnati, Ohio) according to the manufacturer instructions. Integrity of RNA was confirmed by observing bands on an agarose gel and concentration was determined by nanodrop. Reverse transcription of RNA was done using ProtoScript II First Strand cDNA synthesis kit (New England BioLabs, NE, E6560L) following the manufacturer protocol, in a MultiGene OptiMax Thermal Cycler (Labnet international, NJ). The qRT-PCR was done with a CFX Connect Bio-Rad system with Perfecta SYBR green supermix (QuantaBio, MA) and gene specific primers for *Defensin, Drosomycin*, *Diptericin, Metchnikowin* and the housekeeping gene *Tubulin* (Integrated DNA Technologies, IA). Cycling conditions for PCR included a denature step at 94 ˚C for 15 seconds, annealing step at 55 ˚C for 30 seconds, and an extension step at 68 ˚C for 1 minute. All steps were conducted for a total of 40 cycles. Fold change was measured

according to the ΔΔCT Method. Experiments were carried out with three biological replicates with plots shown as bar graphs with individual points representing each replicate. Statistics shown as One-way ANOVA done in GraphPad Prism.

pHrodo phagocytosis

Injections were carried out as previously described for *S. pneumoniae* except with a 4 mg/ml suspension of pHrodo Red *E. coli* BioParticles Conjugate for phagocytosis as a substitute for the bacterial solution. This solution was diluted 1:4 in PBS containing either 250 μM of LA, AA, or PGE_2 immediately prior to injection. A negative control of no protein was injected for analysis along with the 3 different protein doses. 3 flies were injected for each treatment group with a total of 3 biological replicates each. Injected flies were incubated at 28°C with 50% CO_2 for 1 hour. After incubation, the dorsal side of the abdomen of the flies was imaged with an X-Cite® 120Q fluorescence lamp, and a ZEISS Axiocom 506 Color microscope camera attached to a ZEISS SteREO Discovery V12 microscope at 10x magnification. ImageJ software was used to measure area-normalized corrected total fluorescence of isolated red channels. Statistics were shown as one-way ANOVA, with error bars depicting mean with SEM.

Lipidomic analysis – Lipotype

For the analysis of PUFA-derived lipid mediators and metabolites, the following sample preparation steps were performed. A total of 500 μL of plasma was mixed with a combination of antioxidants and an internal standard comprising of: 14,15-DHET-D11, 15-HETE-d8, 20-HETE-d6, 8,9-EET-d11, 9,10-DiHOME-d4, d4-12(13)-EpOME, d4-13-HODE, d4-PGB2, d4-PGE2-13,14-dihydro-15-keto, d4-PGF2a, LTB4-D4, and PGE2-

D4, with a concentration of 1 ng each (obtained from Cayman Chemical, Ann Arbor, USA). To induce protein precipitation and alkaline hydrolysis, methanol and sodium hydroxide were added to the sample, which was then incubated at 60°C for 30 minutes. After centrifugation and pH adjustment, the resulting supernatant was applied to Bond Elute Certify II columns (Agilent Technologies, Santa Clara, USA) for solid phase extraction. The eluate from the columns was evaporated using a heating block at 40°C under a stream of nitrogen, resulting in a solid residue. This residue was dissolved in 100 µL of methanol/water. Subsequently, the samples were subjected to liquid chromatography-tandem mass spectrometry (LC/ESI-MS/MS) analysis. An Agilent 1290 HPLC system equipped with a binary pump, multisampler, and column thermostat was utilized, employing a Zorbax Eclipse plus C-18 column with dimensions of 2.1 x 150 mm and a particle size of 1.8 µm. A gradient solvent system consisting of aqueous acetic acid (0.05%) and acetonitrile/methanol (50:50, v/v) was used. The flow rate was set at 0.3 mL/min, and the injection volume was 20 µL. The HPLC system was coupled with an Agilent 6495 Triplequad mass spectrometer (Agilent Technologies, Santa Clara, USA) equipped with an electrospray ionization source. Multiple Reaction Monitoring (MRM) was employed in negative mode, with each compound monitored using at least two mass transitions. To assess data quality, the dynamic range of the instrument was established prior to the analysis. Based on this information, the limits of quantification and coefficients of variation for different lipid classes were determined. The limits of quantification generally ranged in the lower picogram (pg) range, varying depending on

the specific analyte. The average coefficient of variation for a complete set of analytes was found to be less than 15%.

Flysick-*seq* analysis of endogenous *D. melanogaster* genes

A transcriptomic resource titled Flysick-*seq* was utilized to determine gene expression in the form of transcripts per million (tpm) [34]. In total there are seven predicted and confirmed endogenous *D. melanogaster* $sPLA_2$ enzymes, but only six of the genes were found in the database. The six $sPLA_2$ genes were combined with the 3 predicted $iPLA_2$ genes to generate a heat map. Two elongase and desaturase genes were also examined. TPM values were acquired for a variety of infections and an uncultured negative control. The difference in tpm was acquired by subtracting the tpm value of the uncultured sample from the specific bacterial infection and timepoint. The final quantification used for the heat map was reported as the logarithm of the difference in tpm. Heat map was generated using GraphPad Prism. Higher expression is shown by increasing red color, lower expression is shown by increasing blue color.

Recombinant protein expression and purification

CG1583 was expressed recombinantly in *E. coli* BL21 DE3 cells in LB media for 24 hours upon IPTG induction. The protein was purified from inclusion bodies using Thermo Scientific™ HisPur™ Ni-NTA Resin through gravity filtration. Refolding of the protein was achieved by 24-hour dialysis against a pH 8.0 buffer containing 20 mM Tris, 1.0 M Urea, 300 mM NaCl, and 5% glycerol. After refolding, the protein underwent another 24-hour dialysis and was subsequently stored in a pH 8.0 buffer containing 20 mM Tris, 300 mM NaCl, and 5% glycerol. Presence of CG1583 was confirmed by SDS-

PAGE, and its concentration was determined using Invitrogen™ Qubit™ Protein and Protein Broad Range (BR) Assay Kits. Finally, the proteins were flash frozen with liquid nitrogen and stored at -80°C.

Mass spectrometry assay

To determine activity preference of an upregulated $sPLA_2$ gene CG1583, 2.0 µgs of the recombinantly expressed enzyme was added to reactions that contained 100 µM of PLPC, PLPA, PLPG, PLPE, or PLPS, 400 µM of surfactant, 2.5 µM of 17:0 LPC and reaction buffer (20 mM Tris and 5 mM CaCl2 buffer pH 8.0). The reaction buffer was used to store the lipids and surfactant. For mixed phospholipid head group reactions, the same conditions were used as previously described for the enzyme, 17:0 LPC, surfactant, and buffer. For the lipid substrate however, all head groups were combined for a total concentration of 100 µM (20 µM for each different phospholipid headgroup). Enzymatic reaction was performed in a 96 well-plate using a Benchmark Scientific H5000-H MultiTherm heating shaker for 1 hr at 28°C. Negative control was reaction buffer only with no protein, and positive control was 0.125 µgs of Enzchek Bee Venom $sPLA_2$ to ensure the reaction is working as intended (these controls were used for all optimization and specificity reactions). Reactions were quenched with methanol/acetonitrile (80/20, v/v), and the samples were analyzed using the HPLC-MS system. Activity was reported as nmols/µg with subtraction of the negative control as background. Experiments were done in triplicate with error bars on the graph representing standard deviation. For determining activity preference for *sn-2* fatty acids, 2.0 µgs of CG1583 was added to a reaction of 100 µM phospholipid substrate, 400 µM surfactant, and 2.5 µM of 17.0 LPC.

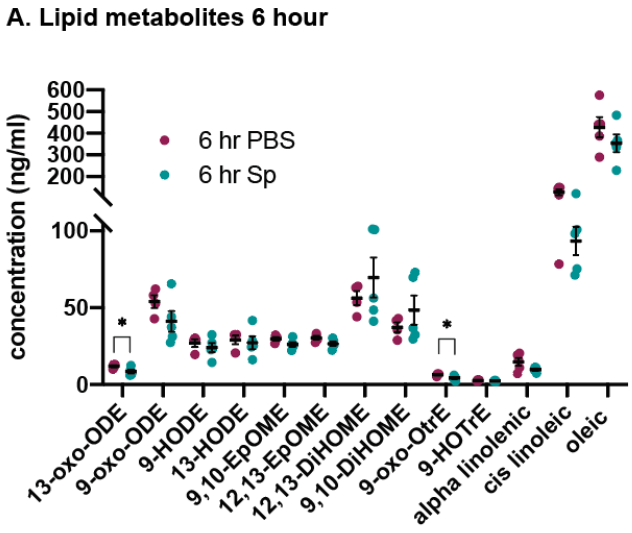
The phospholipid substrate used the experimentally determined preferred phospholipid head group PE. Each lipid substrate had a different *sn-2* fatty acid of either LA, OA, or LLA for a concentration of 100 μM in separate reactions. Enzymatic reaction was performed in a 96 well-plate using a Benchmark Scientific H5000-H MultiTherm heating shaker for 1 hr at 28°C. Negative control was reaction buffer only with no protein, and positive control was 0.125 μgs of Enzchek Bee Venom $sPLA_2$. Mass spectrometry analysis was conducted at the UC Riverside Core Facility. Experiments were done in triplicate with error bars on the graph representing standard deviation.

UCR core facility QQQ lipidomics method

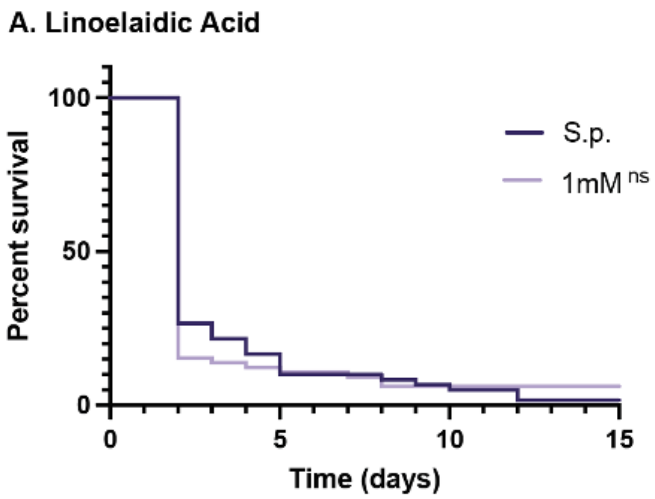
The targeted analysis was conducted using a QQQ XEVO TQ-XS mass spectrometer (Waters Corp., Milford, MA, USA) at the UC Riverside Metabolomics Core. Prior to analysis, the liquid chromatography-mass spectrometry (LC-MS) autosampler was maintained at 4°C. A 2 μL injection volume of the extract was used for analysis. The separation was carried out on a Waters XSelect CSH Phenyl-Hexyl column (3.5 μm, 3.0 × 100 mm, Waters Corp., Milford, MA, USA) with a flow rate of 0.8 mL/min at 30°C. The mobile phase consisted of two components: Mobile Phase A (ACN/water, 95/5, v/v, pH=8.0) containing 25 mM AcNH4, and Mobile Phase B (ACN/water, 50/50, v/v, pH=7.5) containing 25 mM AcNH4. The gradient separation method employed the following time intervals: 0-0.2 min (99% B), 0.2-3.0 min (99% B to 1% B), 3.0-3.8 min (1% B), 3.8-3.9 min (1% B to 99% B), and 3.9-8.0 min (99% B). Data acquisition was performed in multiple reaction monitoring (MRM) mode, with electrospray ionization in positive ion mode. The source and desolvation temperatures were maintained at 150°C

and 600°C, respectively. The desolvation gas flow rate was set at 1100 L/h, the cone gas flow rate at 150 L/hr, and the collision gas flow rate at 0.15 mL/min. Nitrogen was used for all gases except for argon, which was used as the collision gas. The capillary voltage was set at 1.5 kV. To ensure relative abundance, the data was normalized against the internal standard (LPC 17:0). Targeted data processing was performed using the open-source Skyline software [43].

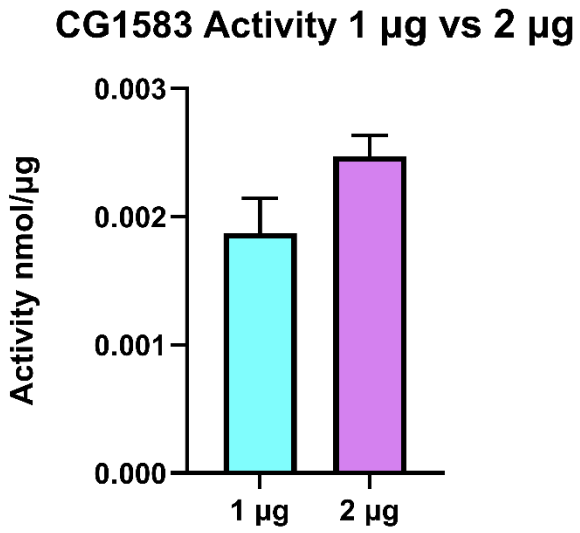
SUPPLEMENTAL INFORMATION



Supplemental Figure S2.1 Hemolymph lipid metabolite panel 6 hours post injection.. At 6 hours after injection, there was a depletion of downstream oxylipins. Flies were injected with 7,000 cells of *S.p.*, and hemolymph was collected for mass spectrometry analysis. In infected flies, significant reductions were observed in 13-oxo-ODE and 9-oxo-OtrE. The experiments were repeated five times, with 200 flies per treatment group per replicate, totaling 2,000 flies. Error bars represent mean ± SEM, and statistical analysis was performed using Welch's t-test.



Supplemental Figure S2.2 Linoleaidic acid survival assay. The geometric isomer of linoleic acid, linoelaidic acid, does not have the ability to rescue a bacterial infection. To demonstrate this, flies were injected with *S.p.* alone or *S.p.* along with 1mM linoelaidic acid, but no difference in the infection outcome was observed.



Supplementary Figure S2.3 CG1583 lipidomics concentration optimization. Enzyme activity of Sc-sPLA$_2$ towards 100 µM of PLPC at two different protein concentrations (1 µg and 2 µg)

STATISTICS

All statistics were done with GraphPad Prism 9.1.0 for Mac. Statistical significance indicated with asterisks indicating the following p-value cut offs: 0.05-0.033*, 0.033-0.002**, 0.002-0.0002*** and <0.0001****.

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Conclusions and Final Remarks

Parasitic nematodes employ diverse mechanisms to enhance their infectivity and survival within host tissues. One crucial mechanism involves the utilization of various excretory-secretory proteins (ESPs) that function as immune effectors. Unraveling the mechanisms underlying these effectors is essential for advancing our understanding of the range of diseases caused by nematodes. Studying the mechanistic action of parasitic nematode ESPs has proven difficult given the technical challenges with establishing an efficient model system that is both viable and safe. In my research, I have addressed this challenge by employing the model insect host *Drosophila melanogaster* and the entomopathogenic nematode (EPN) *Steinernema carpocapsae*. Utilizing EPNs has translational research capabilities due to the high homology with vertebrate parasitic nematodes. Thus, my research can also have an indirect application for generating initial hypothesis on how vertebrate parasitic nematode ESPs may interact in mammalian hosts.

In chapter 1, I attempted to delineate how *S. carpocapsae* modulates host immunity in *D. melanogaster* by characterizing an individual ESP that I named Sc-sPLA$_2$. This protein directly cleaves lipids from the cell membrane lipid bilayer, giving rise to downstream lipids and eicosanoids that have various functions in both insect and mammalian systems. Interestingly, the protein from *S. carpocapsae* exhibits an immunomodulatory effect on *Drosophila*, specifically suppressing the Toll and Imd signaling humoral response, and the cellular immune function of phagocytosis. These effects were linked to the reduction of hemocyte circulation in *Drosophila* upon Sc-sPLA$_2$ treatment. Interestingly however,

the reduction of the hemocytes was not due to cell lysis as shown by the cell lysis assay with S2 cells, a type of hemocyte originating from *D. melanogaster*. Previous studies have linked $sPLA_2$ lethality on cells such as Bee venom $sPLA_2$, to the ability to lyse cells. Elucidating what specific hemocytes are targeted by Sc-$sPLA_2$ and the mechanism for how they are being reduced, would expand the understanding how $sPLA_2$s affect overall physiology in not only insects, but potentially all organisms.

In mammals, downstream eicosanoids such as prostaglandins play a crucial role in immune signaling, influencing pro- or anti-inflammatory responses. Chapter 2 of this work reveals that lipids and eicosanoids can rescue the outcome of infection in *Drosophila*, providing novel insights into lipid-mediated immunity in insect model systems. Specifically, the study in Chapter 2 demonstrates that C18 polyunsaturated fatty acids (PUFAs) are depleted in the fly's hemolymph and can effectively rescue a bacterial infection. These C18 PUFAs are oleic acid (OA), linoleic acid (LA), and alpha-linolenic acid (ALA). Similarly, C20 PUFAs and eicosanoids such as arachidonic acid (AA) and prostaglandins exhibit the same rescuing effect. LA and PGE_2 specifically were able to stimulate phagocytosis, with AA trending towards an increase in phagocytosis after treatment. LA was able to suppress Toll and Imd activation as well. Suppression of Toll and Imd via Sc-$sPLA_2$ can be explained by reduced hemocytes, and thus reduced detection of bacterial pathogens by host cells. In the case of lipids triggering endogenous lipid mediated systems, it is plausible that in a case of increased phagocytosis as early as1 hour post injection, there would be reduced bacteria recognition for Imd and Toll activation by the time the flies were utilized for RNA extraction. There is also the

potential of a negative feedback loop being utilized to not generate a hyperactive immune response in the fly, and ultimately increase the tolerance of the infection to coincide with increased resistance from stimulated phagocytosis.

Overall, this study has established a link between lipid metabolites and cellular immunity in insects. More experimentation is needed however, to validate why Toll and Imd activation seems to be suppressed during lipid mediated immunomodulation. Although this research significantly advances our understanding of nematode effector proteins and *Drosophila* immunity, further investigations are needed to unravel the underlying mechanisms behind these phenomena.