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The tick vector Ixodes scapularis regulates cross-kingdom interactions through innate immunity

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The tick vector Ixodes scapularis regulates cross-kingdom interactions through innate immunity

by
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The tick vector *Ixodes scapularis* regulates cross-kingdom interactions through innate immunity

Fauna Yarza

Abstract

Ticks acquire a bloodmeal from vertebrate hosts, ranging from mammals to reptiles, and are regular interact with a variety of microbes via the bloodmeal host or environment. I set out to characterize ways in which ticks interact with a diverse set of organisms which span biological kingdoms.

One focus of my work was venomous saliva from the tick *Ixodes scapularis* and its influence on multiple processes that are required for successful blood feeding from a variety of vertebrate hosts. Some of these processes include immune evasion through interference of the host's defense system and protecting the tick from harmful microbes. I examined the activity of defensins, a family of antimicrobial peptides, found in tick salivary venom. Studies of tick saliva revealed that defensins are secreted into mice and protect ticks against microbes commonly found on the skin of their host. However, several observations point to a role for defensins that is independent of tick–microbe interactions. Through a collaborative effort, I identified a second, intriguing function where these defensins serve as mast cell activators through an interaction with a G protein-coupled receptor, MRGPRX2. When ticks feed on mice missing an MRGPRX2 ortholog, they have a decrease in weight, suggesting that activation of this receptor benefits ticks during the blood feeding process.

Another area I explored is the complex and incompletely understood interaction between *Ixodes* ticks and *Borrelia burgdorferi,* the causative agent of Lyme disease. In order for ticks transmit *B. burgdorferi*, the bacteria must migrate from the tick's midgut to the salivary glands. This migration process is poorly understood. My exploratory research project suggests that this necessary migration of *B. burgdorferi* is a passive process facilitated by ticks expelling water during blood feeding. Another arm of exploratory research validated previous findings that serum from the western fence lizard *Sceloporus occidentalis,* a common vertebrate host for *I. pacificus,* is bactericidal for *B. burgdorferi.* Both of my exploratory projects are suitable starting points for new research projects which seeks to better characterize cross-kingdom interactions of *Ixodes* ticks. Together, my studies detailed in this dissertation provide a greater understanding of how ticks survive their varied interaction with a diverse group of organisms.

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List of Abbreviations

AMPs = antimicrobial peptides

GPCRs = G protein-coupled receptors

Imd = immune deficiency pathway

MRGPRs = Mas-related G protein-coupled receptors

PAMPs = pathogen associated molecular patterns

PGRPs = peptidoglycan recognition proteins

PRRs = pattern recognition receptors

Chapter 1: Introduction

Ticks are important disease vectors

Ticks are prominent disease vectors in North America. The blacklegged tick, *Ixodes scapularis*, is among the most common tick species in the United States[1]. Ticks from the genus *Ixodes* are hard ticks with four distinct life stages: egg, larvae, nymph, and adult[2]. Upon hatching from the egg, ticks enter the larval stage and require a bloodmeal before they are able to molt into the subsequent nymphal stage. At the nymphal stage, another bloodmeal is required for the nymph to molt into an adult. Upon reaching the final life stage, female and male adults will mate while acquiring their final bloodmeal and females will lay eggs, allowing the lifecycle to continue[2].

As hematophagous arthropods that require blood for feeding, ticks are exposed to a variety of microorganisms during this blood feeding process, including human pathogens. The blacklegged tick is a vector for *Borrelia burgdorferi,* the bacteria that causes Lyme disease. The Lyme disease bacterium is acquired by *I. scapularis* during the larval or nymphal bloodmeal if ticks feed on an infected vertebrate host, typically a small rodent[3]. After acquiring *B. burgdorferi*, these bacteria reside in the midgut of the tick. During the next bloodmeal, *B. burgdorferi* undergoes rapid proliferation in the midgut and disseminates to the salivary glands. Once *B. burgdorferi* is in this specialized tissue, *Ixodes* ticks are then able to transmit the bacteria to their next blood meal host via tick saliva. While we understand the transmission cycle that exposes incidental hosts, like humans or dogs, to *B. burgdorferi* infection, the molecular mechanisms that control the transmission process are poorly understood.

The ability of an arthropod to acquire, harbor, and subsequently transmit a pathogen demonstrates vector competency. One determinant of vector competence among arthropods is

the immune system of the vector, which regulates pathogen persistence and development[4]. Early studies of tick-*Borrelia* interactions identified that vector competency for *B. burgdorferi* is a trait restricted to ticks in the genus *Ixodes*[3]. Studies have demonstrated when non-vector ticks are injected with *B. burgdorferi*, they mount an effective immune response that neutralizes the Lyme disease causing bacteria[5]. The importance of the immune system to vector competency underscores the need to better understand tick immunity when attempting to limit the impact of tick-borne disease.

Antimicrobial peptides are a vital component of tick immunity

Arthropods rely exclusively on an innate immune system[5]. Many inferences about the tick immune system are based on discoveries in better characterized arthropods, like the fruit fly *Drosophila melanogaster*. However, *Drosophila* resides within the subphylum Hexapoda, while *Ixodes* ticks belong to a divergent subphylum Chelicerata, separated by millions of years[6]. The same conserved innate immunity signaling pathways, such as Toll, immune deficiency (Imd), and JAK/STAT, are present in ticks and other arthropods, yet homologs for key members of these signaling networks are missing from tick pathways (**Fig 1.1**)[7]. Despite the absence of some signaling members, these immune pathways are still functional[8].

The Imd and Toll pathways are initiated by the recognition of pathogen associated molecular patterns (PAMPs), including peptidoglycan from the cell wall of bacteria[5,7]. Peptidoglycan recognition proteins (PGRPs) function as pattern recognition receptors (PRRs) and bind peptidoglycan to initiate these pathways[7]. PGRPs from *Drosophila* are associated with specific pathways (either Toll or Imd), however this level of information is unknown for tick immune pathways[3]. Activation of innate immunity signaling pathways causes a signaling cascade to begin. Toll activation leads to the phosphorylation and degradation of Cactus, causing Dif or

Dorsal transcription factors to translocate into the nucleus and initiate transcription of effectors associated with the humoral response (**Fig 1.1**)[5,7]. Imd signaling similarly leads to the phosphorylation and cleavage of a transcription factor, Relish, which is translocated to the nucleus and initiates the transcription of humoral effectors.

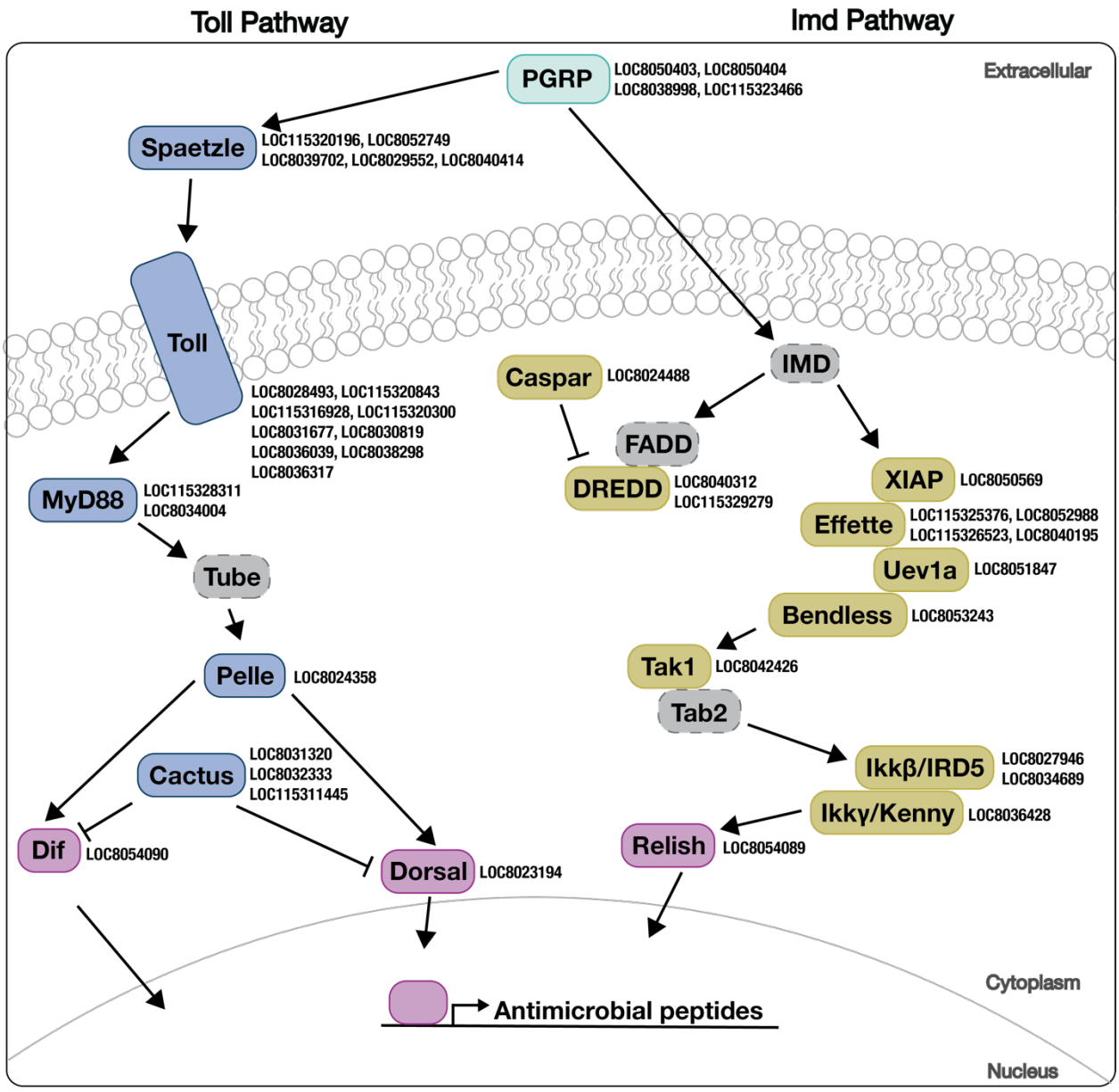

Figure 1.1 A schematic of key genes in the Imd and Toll signaling pathways. This schematic highlights canonical members of microbial recognition and innate immune signaling pathways in *I. scapularis*. A BLAST query provided gene symbols (beginning with LOC) for homologs to these conserved genes in the ISE6 genome. PGRPs (aqua) are represented in the extracellular space because transmembrane PGRPs are absent in ticks. Members of the Toll (blue) and Imd

(yellow) signaling pathways are depicted. Components of these pathways that are not conserved in *Ixodes* ticks are represented in grey. Successful signaling of Toll and Imd pathways leads to activation and translocation of transcription factors (fuchsia) into the nucleus and initiates the transcription of antimicrobial peptides.

The tick immune system is composed of two arms: the humoral response and the cellular response. Cellular responses include phagocytosis by tick immune cells called hemocytes[5]. Humoral responses are defined by secreted effectors, like lysozyme, proteases, and antimicrobial peptides (AMPs)[5]. The ability of the tick immune system to recognize microbes through PAMPs is crucial in initiating the humoral response and increasing gene expression of these humoral effectors. Increased transcription of these effectors in arthropods can also be initiated by non-infectious stimuli, such as physiological changes or environmental stimuli[9]. The benefit of humoral effectors in the absence of microbial challenge is an area where further research is needed.

AMPs are generated as part of the humoral response and are critical for defending arthropods against microbial challenge. Generally, AMPs are small (10-50 amino acids), conserved peptides that can be constitutively expressed or induced downstream of the Toll and Imd pathways[10,11]. These peptides are thoroughly characterized in *Drosophila,* where specific AMPs are associated with either the Toll or Imd pathway[12]. Direct association between AMPs and immune pathways in ticks have yet to be elucidated. Although less information is known about tick humoral activity, studies of AMPs in *Drosophila* are able to contribute to the field of tick immunology because of the high degree of conservation among antimicrobial peptides[11].

One very well conserved family of antimicrobial peptides are those from the defensin family. Defensins are small (<10 kDa), cationic peptides with multiple cysteines that form disulfide bridges[13]. There are two superfamilies of defensins that arose through convergent evolution and are distinguished by structure: *trans*-defensins and *cis*-defensins[13]. *Trans*-defensins

are primarily found in vertebrates and *cis*-defensins can be identified in fungi, invertebrates, and plants[13].

Cis-defensins found in ticks have six conserved cysteines which form 3 disulfide bridges[5]. They are initially translated as a prepro-peptide and are cleaved after a furin motif (RVRR) to generate a smaller, mature peptide[5]. These cationic arthropod defensins are attracted to negatively charged bacterial cell walls[11]. This charged interaction is thought to support defensin antimicrobial activity by allowing these AMPs to accumulate in the membrane of microorganisms and form a pore which lyses the target cell[11]. Defensins are effective antimicrobial agents when tested against Gram-positive bacteria, some Gram-negative bacteria, and some fungi[1]. Tick defensins have been identified in the midgut, hemolymph, ovaries, and salivary glands of various species[14,15]. Focusing on *I. scapularis*, defensins from this tick species are referred to as scapularisins and have undergone a gene expansion[6]. Some scapularisins have been functionally characterized and are effective AMPs against *Listeria* spp. and the pathogenic plant fungi *Fusarium culmorum* and *Fusarium graminearum*[1]. Scapularisins in hemolymph have been evaluated for their ability to inhibit or kill *B. burgdorferi* injected into *I. scapularis* and previous work claims that these AMPs do not target *B. burgdorferi*[5]. However, there remains space for exploring the ability of scapularisins to protect ticks from other microbes that are encountered during the tick lifecycle.

AMPs, including defensins, have been gaining attention for their ability to modulate processes outside of antimicrobial activity[11]. Mammalian defensins from humans can initiate immune cell recruitment and wound healing[16]. Mastoparan, a cationic AMP discovered in wasp venom, is similarly able to promote wound healing and prevent tissue scarring[17,18]. Defensins from the European *Ixodes ricinus* are capable of lysing red blood cells at concentrations above 12.5 uM, suggesting that these AMPs may contribute directly to hematophagous feeding[19]. These findings

indicate that conserved AMPs have functions beyond targeting microbes. Discovering alternative roles of AMPs is an active area of research and future discoveries will reveal alternative functions for AMPs derived across domains of life.

Innate immune pathways and AMPs are conserved features among animals; however, tick immunity shows divergence from well characterized systems in other invertebrates[7]. Despite this difference, ticks have a functioning and responsive immune system. Understanding the interactions between tick immunity and the microorganisms they encounter is a high priority because knowledge about these processes will create new opportunities to break the transmission cycle and limit the burden of tick-borne infectious diseases.

Tick-bacteria interactions

Ticks have intimate and prolonged interactions with microorganisms that they encounter during the feeding process, including both tick-borne pathogens and microbes associated with the hosts ticks feed on. Genetic traits, such as tick immunity, contribute to the outcome of these tick-microbe interactions[20].

Borrelia burgdorferi, the causative agent of Lyme disease, is a bacterium that is closely associated with ticks from the genus *Ixodes*. *Ixodes* ticks are uniquely able to acquire, harbor, and subsequently transmit this pathogen, demonstrating vector competence for this human pathogen. Vector competence is a trait directly influenced by the immune system of an arthropod[20]. Ticks from the genus *Ixodes* acquire *B. burgdorferi* by feeding on an infected vertebrate host, typically a small rodent, at the larval or nymphal life stage. During blood feeding, *B. burgdorferi* undergoes rapid proliferation in the midgut, enters the hemocoel, a body cavity filled with circulatory fluid (hemolymph) and hemocytes (immune cells), and finally disseminates to the salivary glands. This event is necessary for transmission of *B. burgdorferi*,

but less than 0.1% of this tick-borne pathogen in the tick midgut successfully enter salivary glands[21]. The cause of this transmission bottleneck is not yet understood.

There is evidence that some hemocytes in tick hemolymph phagocytose *B. burgdorferi* before they successfully encounter the salivary glands[5]. Additional studies examined how *B. burgdorferi* is affected when incubated with hemolymph from *I. scapularis*. This work found that *B. burgdorferi* viability and activity is not affected by tick hemolymph, suggesting that the humoral immune factors in this fluid may not be a significant contributor to the dissemination bottleneck that *B. burgdorferi* encounters during the transmission cycle[5].

While the transmission cycle of *B. burgdorferi* as a tick-borne pathogen is well known, investigators have yet to identify if harboring *B. burgdorferi* offers the blacklegged tick an evolutionary advantage. Such discoveries have been documented for *I. scapularis* and other tick-borne pathogens. One example is *Anaplasma phagocytophilum,* the causative agent of human granulocytic anaplasmosis. When *I. scapularis* ticks are infected with these bacteria they have an increased cold tolerance[22]. Such examples illustrate how harboring tick-borne pathogens can provide ticks with an evolutionary advantage which may prevent the tick from targeting such organisms via their immune systems.

While *B. burgdorferi* appears to be innocuous to *I. scapularis*, there are other microorganisms which may cause the tick harm during blood feeding. One of the first challenges a tick faces when biting a vertebrate host is the variety of microorganisms comprising the skin microbiome. The skin microbiome is one immune barrier which protects the skin of vertebrates[23]. Gram-positive bacteria make up a large proportion of bacteria in the skin microbiome[23]. Recent work has revealed that a skin commensal, *Staphylococcus epidermidis*, is pathogenic to *I. scapularis*[24]. Dae2, an amidase effector which target bacterial cell walls, is a

component of the *I. scapularis* immune system which has been demonstrated to combat tick infection by *S. epidermidis*[24]. When Dae2 activity is inhibited through gene knockdown or antibody-mediated blocking, ticks injected with *S. epidermidis* have decreased survival compared to ticks with an intact immune system[24]. This data supports the conclusion that ticks can combat tick pathogens via an immune response and this same immune system permits the colonization of tick-borne pathogens. Immune systems in other organisms are known for having components with redundant functions, which suggests that there are additional humoral factors capable of protecting the tick against tick pathogens encountered during the feeding process.

Venomous tick saliva mediates interactions with vertebrate hosts

Venom is defined as "a secretion, produced in a specialized gland … delivered to a target animal through the infliction of a wound … contain[ing] molecules that disrupt normal physiological or biological processes so as to facilitate feeding or defense by the producing animal."[25,26] Ticks are seldomly acknowledged as venomous creatures although their saliva satisfies this definition of venom based on an analysis of evolutionarily conserved traits among venomous animals[26]. Venomous tick saliva is produced in the salivary glands and secreted into the host animal through a wound created by tick mouthparts. Components of venomous tick saliva suppress normal immune signaling in the host animal and this targeting of the immune system is critical for venom function[25,27].

Tick gene expression changes during blood feeding and generates different salivary components over time that are secreted into the vertebrate host to mediate distinct processes, a process known as sialome switching (**Fig 1.2**)[27]. Early in tick feeding, salivary peptides create a cement cone that anchors ticks in host skin and establishes a site where saliva is injected into the host and ticks can imbibe blood from their host[28]. As a tick feeding continues, new salivary

compounds are secreted into the host in order to actively suppress the immune system. One *I. scapularis* salivary protein that fills this role is Salp15. Salp15 modulates the host immune response by inhibiting proliferation of and signaling by $CD4^+$ T cells[29]. Towards the end of the blood feeding time course, ticks will introduce a different cocktail of salivary effectors into their host to induce physiological changes necessary for the final stages of feeding, including promotion of wound healing[30]. The mechanistic process of wound healing associated with feeding by hard ticks is unknown. However, studies have shown that *I. scapularis* saliva can stimulate fibroblasts, a cell type involved with collagen and extracellular cellular matrix production in the dermis, which may contribute to this process [31].

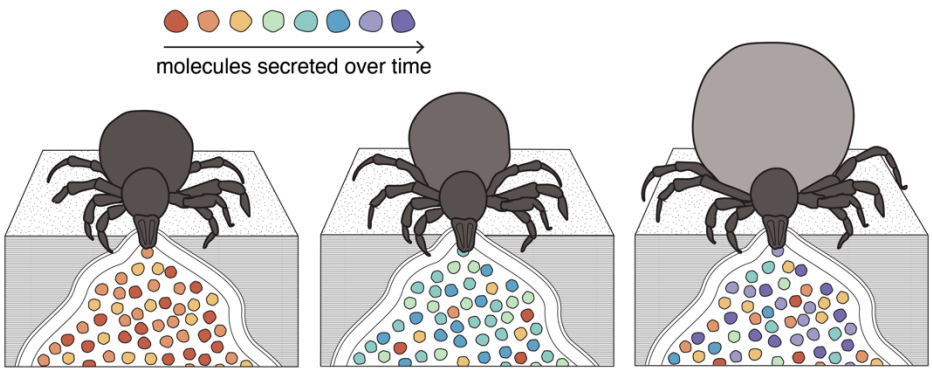

Figure 1.2 Generalized depiction of sialome switching during tick feeding. Schematic showing sialome switching, the dynamic expression and secretion of different tick salivary compounds into the host during different feeding stages.

The conservation among proteins and peptides in venom from different species enables studies from other groups of venomous animals to inform tick research[25,26]. Research on venom has revealed that venomous toxins often undergo significant gene duplication. One explanation for this is that gene duplication allows animals to increase toxin expression which leads to higher doses of toxins that are delivered to their target animal[26]. Another perspective views gene expansion as a method to increase antigenic variation and avoid immune detection by the target animal[25]. Both concepts may be true for tick biology, especially the need for ticks to generate

antigenic variation and avoid the adaptive immune response of their host animal when blood feeding for multiple days.

Studies characterizing peptides from other venomous animals can further inform ongoing research evaluating tick-host interactions. One growing field of research investigates AMPs that are present in venom. Cationic peptides, including defensins, are a well conserved component of animal venom[25,26]. Many cationic peptides function beyond their canonical antimicrobial activity. One example is the cationic peptide mastoparan from the wasp *Vespula lewisii* which is antimicrobial and activates mast cells[32]. Mastoparan activation of mast cells has been shown to recruit neutrophils and dendritic cells to the site of mast call activation, boosting antimicrobial activity and further recruiting immune cells that promote wound healing[18]. Additionally, venom from scorpions, a type of arthropod, share conserved components of venom found in tick saliva that are known to activate mast cells[25]. These discoveries encourage research into the interaction between venomous tick saliva and mast cells, an immune cell type ticks are likely to encounter in skin during blood feeding.

The interaction between tick and the host animal is not a scenario where one animal is a clear victor. This complex interaction promotes gene duplication and diversity of venomous toxins on the tick side. It also promotes an alert immune system capable of detecting and effectively responding to toxins on the host side. Ultimately, the molecular interactions between active components of venomous tick saliva and the host animal is an arms race where there is an ongoing competition between both parties.

Mast cells are sentinels of vertebrate skin

Mast cells are first responders for the immune system in skin[18]. These cells can respond to stimuli from parasites, pathogens, and allergens in seconds to minutes[33]. Mast cells have been

best characterized for their role in allergic reactions mediated by IgE activation through binding the high affinity FcεRI receptor on the mast cell surface[33]. Mast cell activation causes degranulation, where mast cells release a number of preformed mediators including heparin and histamine[33]. Once mast cells are activated, they also begin to produce cytokines that recruit immune cells to the site of activation, including neutrophils and dendritic cells[33]. Activation of mast cells, recruitment of immune cells, and the activity of preformed compounds in mast cell granules all play a role in clearing pathogens or parasites[33].

Studies in recent years have begun to characterize alternative mast cell activation pathways. This cell type has a variety of PRRs on its surface[18]. Non-IgE PRRs are responsible for mast cell activation by specific substances. One example is a non-IgE mediated activation of mast cells by scorpion venom[25]. Other studies of venom-mast cell interactions reveal that peptides in venom activate a G protein-coupled receptor, MRGPRX2[18]. These studies have created an opportunity to expand the field of venom-mast cell interactions to include tick venom, which are often overlooked as venomous creatures that target the immune system of their hosts.

MRGPRX2 is a mast cell receptor that interacts with cationic peptides

G protein-coupled receptors (GPCRs) are a large family of receptors which contribute to cell signaling in vertebrates[34]. This family of receptors is distinguished by a seven transmembrane domain which bind an extracellular ligand and undergo a conformational change to initiate an intracellular signal through interaction with a G protein[17,34]. There are many subfamilies of GPCRs[17]. One subfamily which has been the focus of recent studies are the Mas-related G protein-coupled receptors (MRGPRs)[17]. Expression of MRGPRs was initially identified in neurons and these receptors were initially proposed to have a role in itch and pain[17,35]. There are four MRGPRs (MRGPRX1 – MRGPRX4) identified in humans[36]. Additional

work identified these receptors on the surface of innate immune cells, including mast cells and possibly eosinophils, basophils, and keratinocytes[17,37,38]. The immune cells expressing these receptors are cell types that ticks are likely to encounter when feeding on a vertebrate host through their skin.

Research focusing on non-IgE mediated activation of mast cells has begun exploring mast cell activation via MRGPRX2. MRGPRX2 activation is caused by a variety of cationic molecules called basic secretatgogues[39]. Basic secretagogues are defined as substances with a cationic charge and hydrophobic regions[40]. Many cationic peptides are basic secretagogues and have the ability to activate mast cells via MRGPRX2[38]. Activation by basic secretagogues also occurs in the mouse ortholog for MGRPRX2, Mrgprb2[18]. One example of cationic peptides capable of activating MRGPRX2 are vertebrate β-defensins, a defensin superfamily that function similarly to arthropod defensins but arose through convergent evolution[13]. Human β-defensin stimulation of MRGPRX2 has been proposed to function as a positive feedback loop because these AMPs are secreted by neutrophils after being recruited by cytokines produced *de novo* from activated mast cells[18,41]. Another example of an AMP that activates MRGPRX2 can be found in wasp venom. Mastoparan, a cationic AMP in wasp venom, activates MRGPRX2 and initiates the recruitment of neutrophils and CD301b$^+$ dendritic cells to the site of activation[18]. The combination of antimicrobial activity from mastoparan and recruited neutrophils, in addition to the role of CD301b$^+$ dendritic cells promoting wound healing, have advanced the concept of using MRGPRX2 activating peptides in a therapeutic setting to promote wound healing[18].

The growing body of evidence demonstrating that cationic peptides, including AMPs from venom, initiate mast cell activation through MGRPRX2 encourages future studies of this receptor's interaction with peptides from tick venom. Mast cells mediate many processes that

ticks may manipulate and take advantage of late in feeding. These mast cell-initiated processes include increasing vascular permeability through the release of histamine in mast cell granules, a process which may improve tick feeding by increasing blood flow to the bite site[18,41]. As seen in the characterized mastoparan-MRGPRX2 interaction, activation of this receptor promotes recruitment of wound healing CD301b^{+} dendritic cells[18]. The influence of MRGPRX2 activation on wound healing may contribute to a tick's ability to completely heal the wound created during blood feeding[30]. Given the role of mast cells expelling parasites from tissues, activation of mast cells by cationic peptides secreted late in feeding may be one tool ticks use to promote detachment from the cement cone that firmly adheres them to the skin of their vertebrate host[30,42]. Continuing to study the effect of conserved peptides in tick venom on mast cells will identify findings that are translatable to other venomous creatures and reveal new ways in which ticks are able to molecularly manipulate the hosts they feed on.

Chapter 2: *Ixodes scapularis* salivary defensins have antimicrobial activity against vertebrate skin commensal bacteria

Introduction

Ticks are important disease vectors and *Ixodes scapularis*, the blacklegged tick, is the most prominent tick species in the United States[1]. *Ixodes* ticks are hard ticks which require a bloodmeal to advance to the next stage in their life cycle[2]. While ticks are recognized for their ability to transmit tick-borne diseases, there is a limited understanding of how the tick innate immune system interacts with microbes encountered during the blood feeding process. These microbes include tick-borne pathogens, bacteria found in the environment, and microbes on the skin of vertebrate hosts. Within the last decade, significant molecular biology advances have enabled the developing field of tick immunology to increase the understanding of these tick-microbe interactions[43,44]. One example underscoring the importance of the tick immune system is the discovery that skin commensals from vertebrate hosts are pathogenic to *I. scapularis* ticks and an amidase in tick saliva combats these microbes[24]. Additional studies have verified that components of venomous tick saliva mediate many processes in the host that support ticks during blood feeding, including antimicrobial defense[3].

Defensins are conserved cationic AMPs found in the venom of chelicerates, a phylogenetic group including ticks, spiders, and scorpions[25]. This family of AMPs is characterized by a domain containing six cysteines and a cationic charge at physiological pH[45]. These AMPs are effective at combatting Gram-positive bacteria, some Gram-negative bacteria, and fungi through lytic activity[13]. Defensins from *I. scapularis* are referred to as scapularisins. Earlier work evaluated the ability of scapularisins to provide immune protection against *B. burgdorferi*, the causative agent of Lyme disease transmitted by *I. scapularis,* and found that

these AMPs did target *B. burgdorferi*[5]. However, *B. burgdorferi* is just one microbe that *I. scapularis* encounters and is not indicative of all scapularisin-microbe interactions.

Two scapularisins have been functionally characterized *in vitro* to have anti-fungal and anti-*Listeria* activity, however, these findings are not particularly informative when thinking about tick-microbe interactions in nature[1]. This same work used RT-PCR to identify that these scapularisins are expressed in the salivary glands of *I. scapularis*[1]. Additional studies have characterized the proteome of *I. scapularis* saliva during tick feeding and did not detect scapularisins, possibly due to limitations of the mass spectrometry method used and the small size of mature defensin peptides[30]. These previous studies set the stage to address unanswered questions about the presence of scapularisins in venomous tick saliva and whether these AMPs execute their canonical activity to protect ticks against microbes encountered in nature, particularly pathogens of ticks.

I am interested in addressing these open questions about immune protection provided by scapularisins and the presence of these AMPs in tick saliva. Microscopy and immunoblotting enabled detection of scapularisins in tick salivary glands and saliva. I further assessed the ability of scapularisins to protect ticks against common skin commensal bacteria which can act as pathogens of ticks and found these scapularisins do provide ticks with immune protection.

Results

The *Ixodes scapularis* genome contains 12 unique scapularisins

Ticks inject a diverse mix of molecules into the wounds they create during blood feeding. Venomous tick saliva contains hundreds to thousands of polypeptides which are introduced to the host at different times to evade and suppress host immune responses[27]. The introduction of these salivary components at different times, known as sialome switching (**Fig 1.2**), enables the

tick to facilitate distinct processes required for successful blood feeding[28]. The tick immune system is also represented in the tick sialome, including secreted proteins that protect ticks during their intimate and dynamic interactions with microbes as a bloodmeal is acquired[24]. Given the variety of AMPs with specialized functions that are found in the venom of other chelicerates, I hypothesized that scapularisin AMPs may have previously uncharacterized functions[10,16,25]. To begin investigating this possibility, I set out to identify all scapularisins in the ISE6 genome using TBLASTN with the conserved arthropod domain as a query[46]. I identified 12 unique scapularisin genes containing the conserved arthropod defensin domain with six cystines (**Fig 2.1**). The mature peptide of all 12 scapularisins have a positive charge characteristic of defensins (**Fig 2.1**). A gene expansion event is likely responsible for the multiple unique scapularisins that have been retained in the *I. scapularis* genome[6].

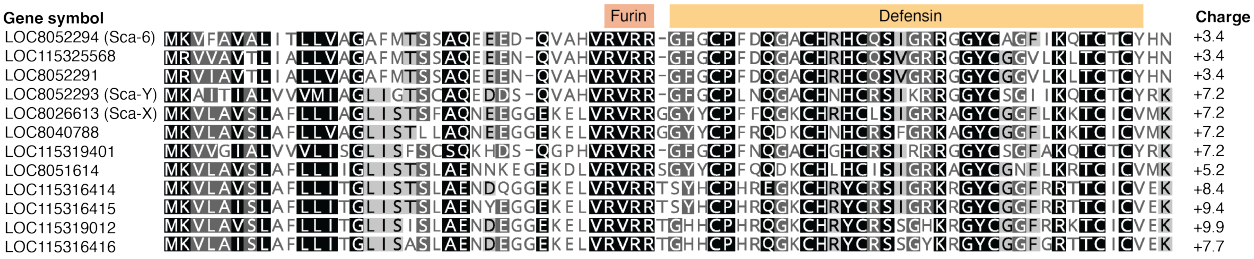

Figure 2.1. The *Ixodes scapularis* genome contains 12 unique scapularisins. Multiple sequence alignment of 12 full length scapularisins. The darker colors indicate highest conservation at each position with black representing 100% conservation. The furin motif (RX(R/K)R) is indicated above the alignment. All amino acids following the furin motif compose the mature peptide wherein the arthropod defensin domain is conserved. The mature peptide is associated with activity of defensins. The positive charge associated with each mature peptide was calculated at a pH of 7. Positive charges range from 3.4 – 9.9.

Scapularisins are present in tick salivary glands and secreted into host animals through venomous saliva

AMP expression is often induced when and where these peptides function[47]. Since AMP localization is often related to activity, I was interested in determining the tissue specificity of scapularisins to learn more about their function. I chose three scapularisins to study in more

detail: LOC8052294 (Scapularisin-6, Sca-6), LOC8026613 (Scapularisin-X, Sca-X), and LOC8052293 (Scapularisin-Y, Sca-Y) (**Fig 2.1**). Moving forward, these scapularisins will be referred to as Sca-6, Sca-X, and Sca-Y, respectively. Sca-6 was identified before the *I. scapularis* genome was publicly available and has been characterized as an effective AMP against some human pathogens[1,44]. However, studies with Sca-6 did not focus on the role of this AMP in tick biology. Sca-X and Sca-Y were annotated as defensins in the ISE6 genome and are functionally characterized in this work for the first time[46]. Each of these full length scapularisins has a high likelihood of being secreted based on SignalP prediction (0.9998)[48]. This was an important trait to evaluate when choosing scapularisins for characterization because AMPs canonically function extracellularly.

I next wanted to assess the presence of scapularisins in tick salivary glands because these specialized organs are where ticks produce venomous saliva containing a variety of compounds responsible for mediating processes in the bloodmeal host[25]. If scapularisins were present in this tissue, they may contribute to influencing processes at the bite site. I hypothesized that scapularisins would be detected in the salivary glands and venomous saliva of *I. scapularis*. To assess scapularisin localization, I used immunofluorescence microscopy to determine localization in antibody-stained salivary glands from unfed adult females (**Fig 2.2**). Sca-6, Sca-X, and Sca-Y are all present in the salivary glands. Interestingly, each scapularisin has a unique localization in this tissue. Sca-6 is relatively well distributed, Sca-X localizes to the periphery of each salivary gland acini, and Sca-Y forms very small puncta in this specialized tissue. Localization of these scapularisins in the salivary glands is consistent with previous work which identified the expression of Sca-6 in salivary glands through RT-PCR[1].

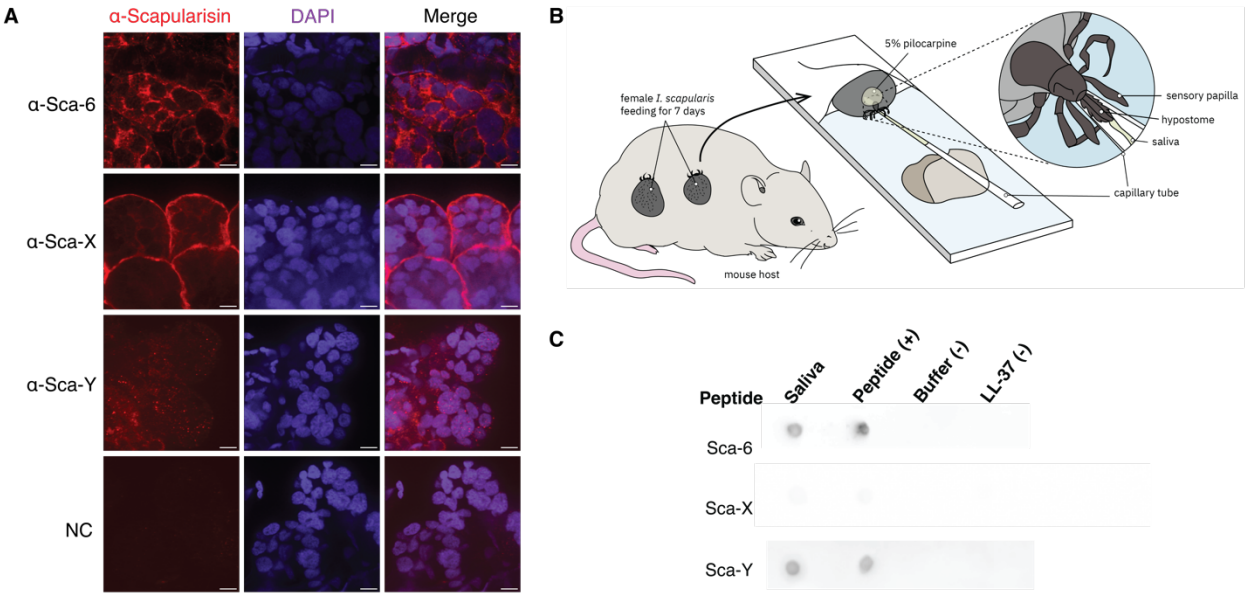

Figure 2.2. Scapularisins are present in tick salivary glands and secreted into host animals through venomous saliva. (A) Confocal fluorescence images of dissected salivary glands from unfed *I. scapularis* females. Panels represent salivary glands stained with pre-immune control serum (NC) or antibodies raised against the mature peptide (Sca-6, Sca-X, and Sca-Y). All samples were stained with DAPI and merged images are shown. Scale bars: 10 µm. Microscopy of salivary glands reveals salivary gland localization of all three scapularisins. (B) Schematic of experimental protocol for venomous saliva collection from *I. scapularis* females. (C) Dot blot detects Sca-6, Sca-X, and Sca-Y in venomous saliva collected from adult *I. scapularis* females. Commercially synthesized mature scapularisins were used as a positive control (+). Negative controls (-) included the low salt buffer synthesized peptides were solubilized in and LL-37, a human derived antimicrobial peptide.

Having assessed scapularisin localization in the salivary glands of female ticks via microscopy, I wanted to further assess if these peptides are secreted into vertebrate hosts through the tick's venomous saliva. I utilized a method for collecting venomous saliva from partially fed ticks (**Fig 2.2**). After seven days of attachment to a mouse, female ticks were forcibly removed, treated with pilocarpine to induce salivation, and saliva was collected with a capillary tube. Using saliva from this collection method, we screened for the presence of Sca-6, Sca-X, or Sca-Y. A dot blot was chosen as the immunoblotting technique for this experiment due to the small size of the mature scapularisins (4.0 – 4.2 kDa), which are difficult to resolve in a traditional western blot. Saliva and controls were pipetted directly onto a nitrocellulose membrane and

stained with antibodies (**Fig 2.2**). Through this approach, I detected Sca-6, Sca-X, and Sca-Y in the venomous saliva of *I. scapularis* females collected after seven days of feeding on mice. Therefore, I concluded that Sca-6, Sca-X, and Sca-Y are expressed in salivary glands and present in venomous saliva that is secreted into vertebrate hosts.

Scapularisins are antimicrobial against skin commensals

Having recognized that scapularisins are among the salivary compounds secreted into the vertebrate host during blood feeding, I wanted to verify the canonical antimicrobial activity of these AMPs. I hypothesized that scapularisins would kill common skin commensals because ticks feed through a wound in the skin and our laboratory previously identified skin commensals as a target of the tick immune system[24]. Furthermore, our lab identified *S. epidermidis,* a common skin commensal, as pathogenic to ticks, which suggests that the tick immune system may have redundant mechanisms to combat these microbes[49].

To assess the antimicrobial activity of scapularisins, I utilized a minimum inhibitory concentration (MIC) assay with modifications specific for cationic AMPs[50,51]. Each scapularisin was tested against 3 different skin commensals from the genus *Staphylococcus*: *S. epidermidis,* a common human skin commensal, *S. hominis*, a skin commensal found on human and animals; and *S. xylosus*, a skin commensal of rodents and other animals[24,52,53]. These tests used 10-fold dilutions between a range of 5 - 0.005 uM. The MIC was determined by growing bacteria in the presence of each peptide concentration and determining the lowest concentration where visible growth was inhibited (**Fig 2.3, Fig 2.4**). I found that all scapularisins were effective AMPs at the concentrations tested and inhibited the growth of these *Staphylococcus* species (**Fig 2.3**, **Fig 2.4**). Sca-6 was inhibitory between 0.5 – 0.05 uM. This finding for Sca-6 is in contrast to previous work which characterized Sca-6 as being unable to inhibit the growth of *S. epidermidis*[1]. This

difference in reported inhibitory activity may be attributed to the methods used in each study, as our protocol utilized a MIC method optimized for cationic peptides[50]. Sca-X was inhibitory between 5 – 0.05 uM, depending on the bacteria being challenged. Sca-Y was the most effective AMP tested against *S. hominis* and *S. xylosus* with an MIC ≤0.005 uM.

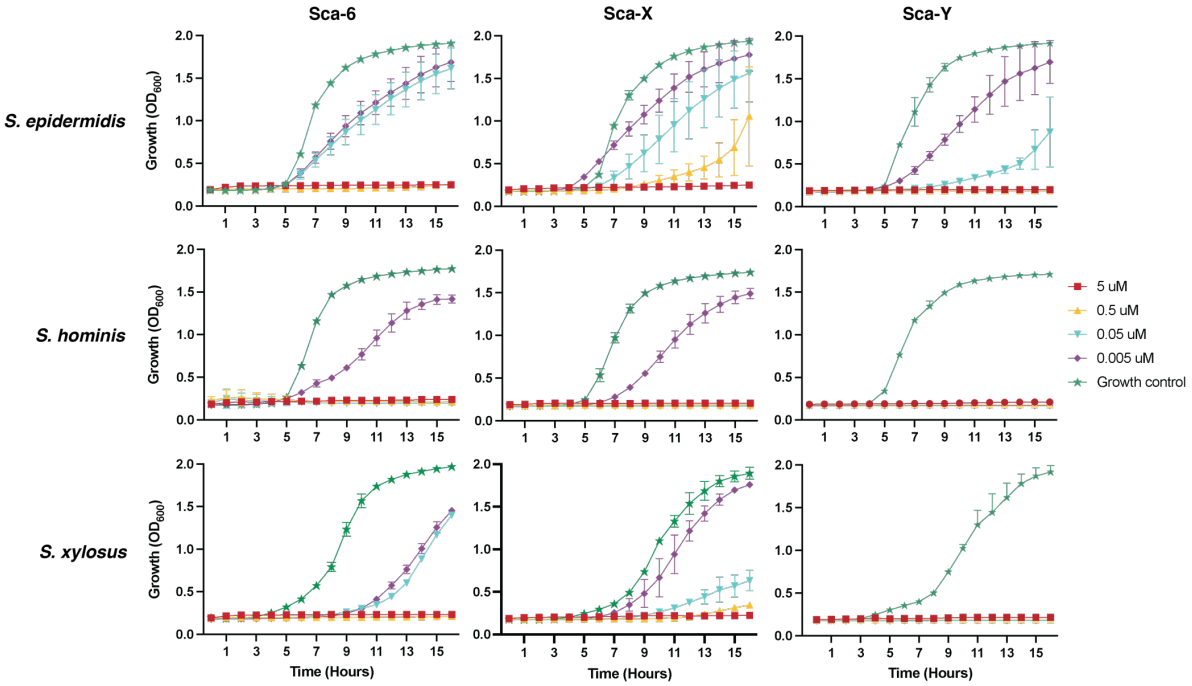

Figure 2.3. Growth curves reveal dose dependent inhibition of *Staphylococci* in the presence of scapularisins. Data from minimum inhibitory concentration (MIC) experiments using a broth dilution method to test Sca-6, Sca-X, or Sca-Y against skin commensals from the genus *Staphylococcus* to determine the lowest concentration of the peptides which prevents bacterial growth. Each point represents the mean at that time and error bars represent 1 SD (n=4).

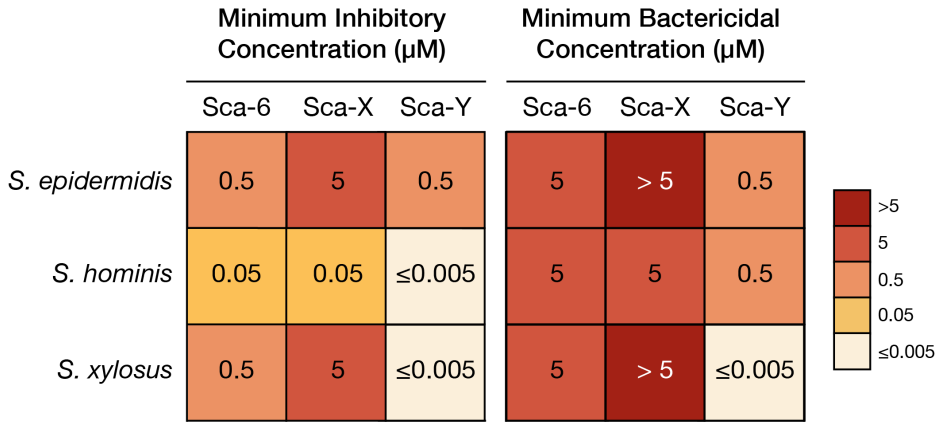

Figure 2.4. Scapularisins inhibit and kill skin commensals *in vitro*. Summary of data from minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) experiments using a broth dilution method to test Sca-6, Sca-X, or Sca-Y against skin commensals from the genus *Staphylococcus*. These experiments determined the lowest concentration of peptides which prevents or kills bacteria tested. MBCs reported as >5 uM were not bactericidal at the highest tested peptide concentration.

In addition to the MIC assays, I completed minimum bactericidal concentration (MBC) assays to determine if these scapularisins could both inhibit and kill *Staphylococcus* skin commensals at the concentrations tested (5 uM - 0.005 uM). These experiments were completed in tandem with the MIC assays as cultures from the completed MIC assays were stamped onto Mueller-Hinton agar for MBC determination[51]. The MBC was determined by identifying the lowest concentration tested which prevented the growth of bacterial colonies (**Fig 2.4**). Sca-6 was bactericidal against all 3 *Staphylococcus* species at 5 uM. Sca-X bactericidal activity was limited to *S. hominis* at 5 uM. Both *S. epidermidis* and *S. xylosus* were able to grow colonies after exposure to 5 uM Sca-X. Similar to the results from MIC determination, Sca-Y was very effective at killing these *Staphylococcus* species with a MBC of 0.5 uM for *S. epidermidis* and *S. hominis* and a MBC ≤0.005 uM for *S. xylosus.* These data indicate that Sca-6, Sca-X, and Sca-Y are effective AMPs and have the potential to protect ticks against vertebrate skin commensals which function as tick-borne pathogens.

Discussion

Previous studies suggested that scapularisins did not offer immune protection for *I. scapularis*, however, these experiments focused on the effect of scapularisins on the tick-borne pathogen *B. burgdorferi* which is innocuous to the tick[5]. The work described above demonstrates that scapularisins function as part of the tick immune system and protect *I. scapularis* from tick pathogens found on the skin of vertebrate hosts. Additionally, this work verifies that scapularisins are secreted in *I. scapularis* venomous saliva which localizes these AMPs to the

bite site where they can execute their canonical antimicrobial activity. These findings are in line with previous work that identified components of tick saliva which combat host skin commensals and demonstrates that tick immunity has redundant mechanisms to combat these tick pathogens[24].

The scope of this work focused on functionally characterizing three of the twelve unique scapularisins in the ISE6 genome. One way that the scapularisin gene expansion may benefit *I. scapularis* is by creating antigenic diversification, enabling ticks to avoid the adaptive immune system of their vertebrate host during the multiple day blood feeding process. If this is true, then all twelve scapularisins may offer comparable immune protection by targeting similar groups of microbes. It is also possible that the remaining scapularisins have novel functions in tick biology, which should be addressed, as defensins in chelicerate venom can have a variety of functions, such as targeting ion channels when introduced to host or prey animals[25,26].

This characterization of scapularisin immune activity against host skin commensals provides additional information about how ticks manage interactions with organisms that span biological kingdoms. Future work should explore the ability of scapularisins to target non-*Staphylococcus* skin commensals. Additionally, the recent adaptation of CRISPR enabling genetic manipulation in ticks permits future studies to assess how individual scapularisins or the entire family of scapularisins affect tick biology[43]. These studies would provide the field of tick immunology with a more complete understanding of the impact scapularisins have during the blood feeding process and may encourage additional studies of AMPs found in venomous tick saliva[30].

The findings described create a new understanding of how scapularisins affect tick biology by verifying that they offer immune protection against known tick pathogens. This work

demonstrates that the relatively simple tick innate immune system is able to selectively target bacteria through antimicrobial activity. As the field of tick immunology expands, future work may lead to modifications or manipulations of the tick immune system that can prevent the transmission of tick-borne diseases by altering which microbes are targeted by components of the tick immune system.

Methods

Multiple sequence alignment

Full length peptide sequences were pulled from the ISE6 cell line genome (GenBank accession GCA_002892825.2) after being identified via TBLASTN with the conserved arthropod defensin domain as a query. These sequences were input into Geneious Prime. A multiple alignment was done using Geneious Alignment (Alignment type: global alignment with free end gaps, cost matrix: Biosum62) with default options.

Determination of positive charge for each mature scapularisin

The amino acid sequence for each peptide after the furin motif was entered in Protein Calculator v 3.4 (http://protcalc.sourceforge.net/) to determine charge at a pH of 7.0.

SingalP

Full length peptide sequences were input into SignalP 6.0 (https://services.healthtech.dtu.dk/service.php?SignalP). Settings were as follows: organism Eukarya, output long format, model mode fast.

Antibody generation

Polyclonal antibodies specific to Sca-6, Sca-X, and Sca-Y were raised from rabbits by Thermo Scientific using synthetic peptides corresponding to the following peptide antigens: Sca-6: GCPFDQGACHRHCQSIGR, Lot: PWG6714; Sca-X: GRRAGYCGGFLKKTCICVMK, Lot: PWJ6854; Sca-Y: GACHNHCRSIKRRGGYCSG, Lot: PXA6964. Affinity purification from rabbit serum was used for the Sca-6 and Sca-Y antibodies. The antibody for Sca-X is contained in a total IgG fraction from serum due to difficulties with affinity purification.

Whole mount immunostaining

The immunohistochemistry imaging of tick salivary glands was performed as previously described with modifications[24]. Salivary glands from unfed and uninfected *I. scapularis* females were dissected and fixed in formalin for 3 days at room temperature. Fixed glands were first dehydrated in 100% methanol and rehydrated in PBS with 0.1% Tween20. Salivary glands were washed 3 times in PBST (PBS with 0.3% Triton X-100) for 15 minutes, the glands were incubated with blocking buffer (PBST with 10% fetal bovine serum, Sigma) for 30 minutes at 37°C. Four primary stains were diluted in the blocking buffer: anti-Sca-6 (1:500), anti-Sca-X (1:500), anti-Sca-Y (1:100), pre-immune serum (1:1000), and incubated with the glands overnight at 4°C. After washing 3 times in PBST for 30 minutes, the glands were incubated with secondary antibody donkey anti-rabbit Alexa Fluor 647 (Invitrogen) diluted 1:1000 in the blocking buffer for 1 hour at room temperature. The glands were washed in PBST for 3 times and stained with DAPI (Invitrogen, 50ng/ml in PBST) for 20 minutes at room temperature. After washing 3 times in PBST for 30 minutes, the glands were transferred onto microscope slides and mounted with 10 µl ProLong Diamond Antifade Mountant (Invitrogen). Samples were cured in the dark at room

temperature for 24 hours before fluorescence imaging was performed on a Nikon spinning disk confocal microscope using a 60x/1.40 objective.

Animals

Animal experiments were conducted in accordance with the approval of the Institutional Animal Care and Use Committee (IACUC) at UCSF, which maintains full AAALAC accreditation. C3H/HeJ mice (aged 4-6 weeks) from Jackson Laboratories were used for the tick-feeding experiments unless noted otherwise. UCSF Laboratory Animal Resource Center (LARC) or trained Chou lab personnel performed daily health checks for mice.

Tick feeding

Certified pathogen free *I. scapularis* nymphs and adults were purchased from the tick lab at Oklahoma State University (OSU). Ticks were maintained in a sealed incubator with a cycle of 16 hours light/ 8 hours dark. The incubator was kept at 22 °C and ticks were stored in glass chambers with a relative humidity of 95% (saturated solution of potassium nitrate). Animal experiments were conducted in accordance with the approval of the Institutional Animal Care and Use Committee (IACUC) at UCSF. Ticks were fed on C3H/HeJ mice obtained from Jackson Laboratories unless otherwise noted. 30 ≤ or ≤10 adult female ticks were placed on mice anesthetized with ketamine/ xylazine and mice were immediately placed in a warm, humid cage. Ticks were pulled from anesthetized mice at various points during feeding or collected from mouse cages upon engorgement and detachment from mice.

Tick saliva collection

Female ticks were pulled from mice on day 7 of feeding. Each tick was taped to a glass slide and a total of 5 uL of 5% pilocarpine in methanol was pipetted on the scutum of the tick during a 3-hour window. During this time, a glass capillary tube was placed over the hypostome of each tick to collect saliva. Collected saliva was stored at -80°C.

Synthesized peptides

Sequences for peptide synthesis were obtained from the *I. scapularis* ISE6 cell line genome (GenBank accession GCA_002892825.2): Sca-6 XP_029842170.1, Sca-X XP_002404709.2, Sca-Y XP_029839602.1. Only the amino acids following the furin cleavage motif (RVRR) were chemically synthesized in order to generate mature peptides. Peptides were synthesized with 95% purity using standard procedures (Peptide 2.0). Lyophilized peptides were solubilized in low salt buffer (100 nM NaCl and 20 mM HEPES, pH 7).

Dot blot with venomous saliva

Venomous saliva samples were quantified using a NanoDrop 2000 (Thermo Scientific ND-2000C) and normalized to a concentration of 8 mg/mL. Controls (LL-37 (Anaspec AS-61302) and synthesized peptides) were diluted to a concentration of 500 uM. Samples were pipetted directly onto nitrocellulose membranes (GVS 1212590) and dried at room temperature. All dilutions were prepared in TBST (Tris-buffered saline 0.1% Tween20) and all incubations proceeded for 1 hour at room temperature with a washing step in between. A 5% milk solution was prepared for blocking and membranes were incubated in that solution. Membranes were then incubated in primary stain (Sca-6 and Sca-X antibodies were diluted 1:500, Sca-Y was diluted

1:167). A secondary stain of Goat anti-Rabbit-HRP (Advansta R-05072-500) was used at a 1:5000 dilution. This was followed by chemiluminescence detection with the Azure Radiance Plus Femto reagent (AC2103) on the Azure c400 instrument.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays

MIC experiments were conducted following previously published methods broth dilution for antimicrobial peptides that require the presence of acetic acid/ BSA with omission of solution A[50]. All assays were conducted in polypropylene 96-well plates (Corning CLS3879) at 37°C using the Biotek Synergy H1 Hybrid Multi-Mode Reader. After completion of the MIC assay, a microplate replicator was used to stamp cultures from each well onto Mueller-Hinton agar[51]. Agar plates were incubated at 37°C overnight. The MBC was determined by the absence of bacterial growth at a specific peptide concentration. *Staphylococcus epidermidis* BCM060 and *Staphylococcus hominis* SK119 were generously provided by Dr. Tiffany Scharschmidt (UCSF). *Staphylococcus xylosus* was purchased from ATCC (29971 KL162).

Chapter 3. Scapularisins activate mast cells via the Mas-related G protein-coupled receptor MRGPRX2

Introduction

Vertebrates have conserved innate and adaptive immune responses to protect against hematophagous parasites and venomous creatures, two categories that describe ticks[25,41]. Mast cells are rapidly activated in response to venom as these immune cells function as first responders in the skin. Mast cell activation leads to degranulation and the release of preformed mediators to initiate an inflammatory response in addition to *de novo* synthesis of cytokines which recruit immune cells to the site of activation[25]. This robust immune response combats parasites in an effort to expel them from the host. However, venomous creatures have numerous conserved proteins that target the immune system of hosts to facilitate blood feeding or self defense[25].

While defensins were initially identified for their antimicrobial function, alternative activities have been discovered in recent years. Arthropod defensins in venom impact host ion channels and promote wound healing and tissue repair via mast cell activation[13,25]. Human β-defensins, which are similar to arthropod defensins in function but arose through convergent evolution, have also demonstrated wound healing activity[16]. Such studies of alternative activities for defensins and other venom derived cationic peptides have identified that these peptides interact with mast cells through a G protein-coupled receptor known as MRGPRX2[18].

I was interested exploring alternative functions for scapularisins and determining if this family of AMPs could mediate processes associated with tick feeding at the bite site. This work identified that scapularisins found in venomous tick saliva activate the MRGPRX2 receptor on the surface of mast cells. A mouse model missing Mrgprb2, a MRGPRX2 ortholog, was used in

a tick feeding experiment and revealed a decrease in weight for ticks fed on mice missing this receptor. These studies indicate that scapularisins execute their canonical antimicrobial activity and interact with MRGPRX2 at the bite site, providing a benefit to ticks during blood feeding.

Results

Scapularisins exhibit distinct temporal expression patterns during tick feeding

I was interested in characterizing the gene expression of all 12 scapularisins in the ISE6 genome (**Fig 2.1**) to identify gene expression patterns during blood feeding and determine when scapularisins may be carrying out their function. This was accomplished by using RNA-sequencing to profile expression changes across several key time points (**Fig 3.1**). Studies of defensins from soft ticks show up-regulated expression during bloodfeeding[54]. In line with those findings, I hypothesized that scapularisins from the hard tick *I. scapularis* would also show increased expression over the course of blood feeding. I looked for scapularisins with a significant difference in gene expression between unfed and engorged ticks, expecting to identify genes with highest expression at later stages of feeding[54]. Half of the scapularisins did not have significant expression changes between unfed and engorged timepoints (**Fig 3.2**). Interestingly, Sca-Y is highly expressed in unfed or flat ticks, suggesting that this scapularisin may play an important role just prior to feeding. We've previously identified that Sca-Y is an effective AMP against *Staphylococcus* skin commensals (**Fig 2.3, Fig 2.4**). The early expression of Sca-Y may be due to its utility as an antimicrobial that is beneficial to the tick when first encountering microbes on the skin of their host.

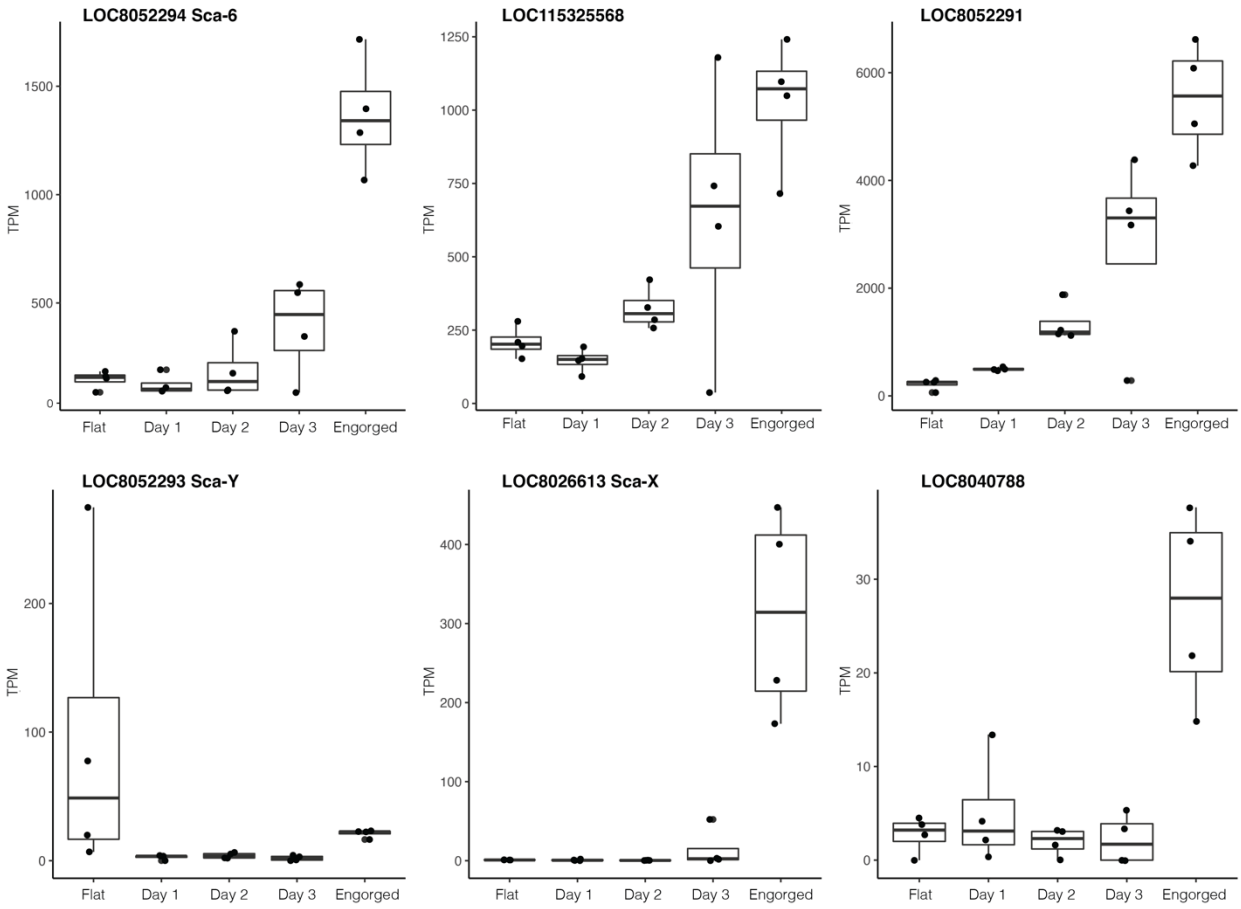

Figure 3.1. **Scapularisins exhibit distinct temporal expression patterns during tick feeding.** Individual plots showing expression of scapularisins with significant gene expression changes between unfed and engorged timepoints (padj <0.05). The Y axis represents transcripts per kilobase million (TPM) during each time point from the nymphal bloodmeal. Each replicate is composed of a pool of five ticks (n = 3-4). Five out of six scapularisins with significant expression changes trend upward as ticks feed and reach engorgement. Sca-Y is the exception where scapularisin expression is highest in flat or unfed ticks, decreases in following timepoints, and shows a modest increase at engorgement.

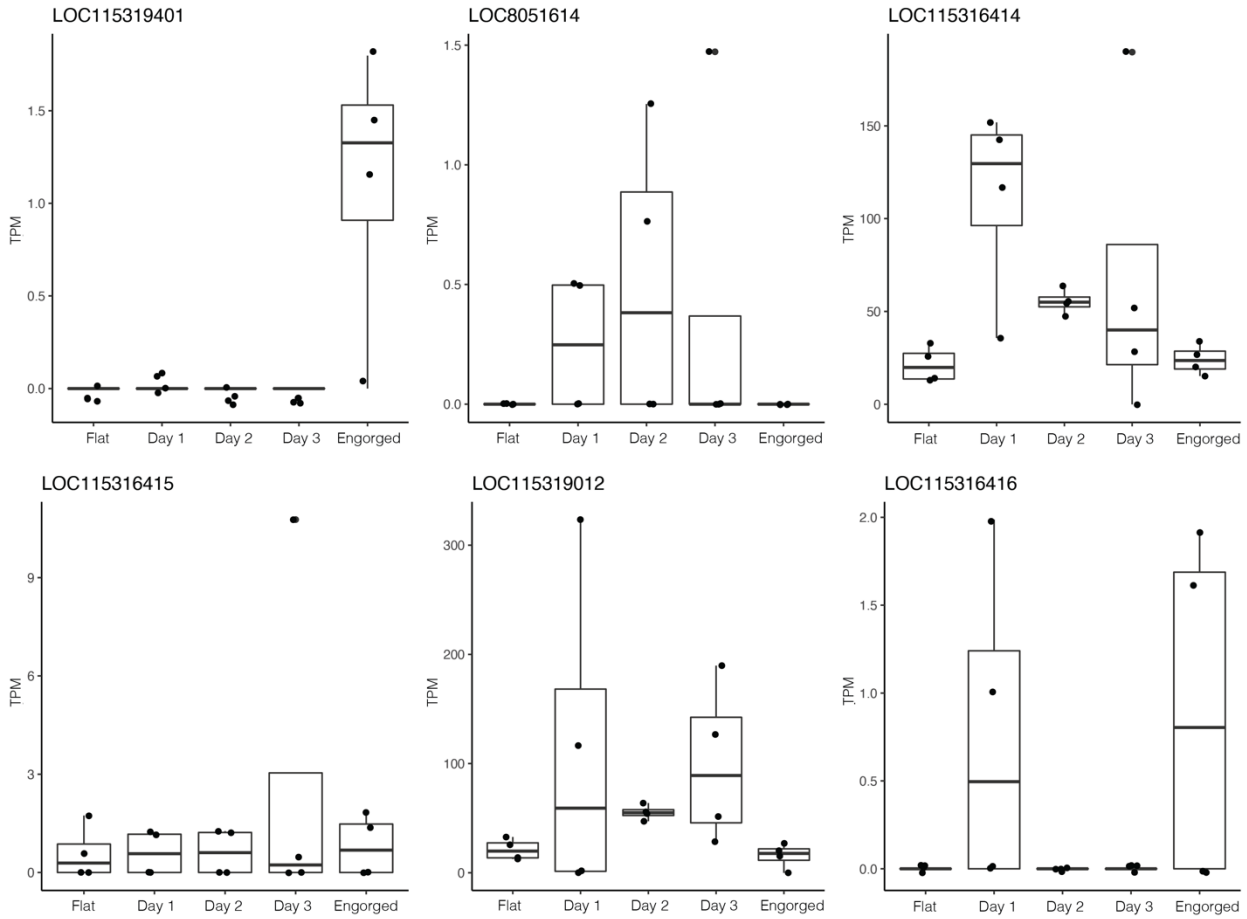

Figure 3.2. Non-significant expression patterns of scapularisins when comparing flat and engorged time points. Expression of scapularisins without significant gene expression changes between flat/ unfed and engorged timepoints (padj <0.05). Plots show transcripts per kilobase million (TPM) during each time point on the Y axis. Each replicate is composed of a pool of five ticks (n = 3-4). These scapularisins do not show a clear trend in expression patterns.

Five of the six remaining scapularisins with significant differences in expression between flat and engorged timepoints showed a significant increase in expression over feeding, reaching a maximum at engorgement in line with our hypothesis (**Fig 3.1**). Sca-6 and Sca-X were included in the scapularisins with increasing expression. While this scapularisin expression pattern is expected based on studies of defensins in another tick species, it remains unclear what function scapularisins have at these later feeding timepoints.

One possibility is that scapularisins function to limit proliferation of tick-borne pathogens inside the tick late in feeding. Studies examining the number of *Borrelia* in *Ixodes* ticks during

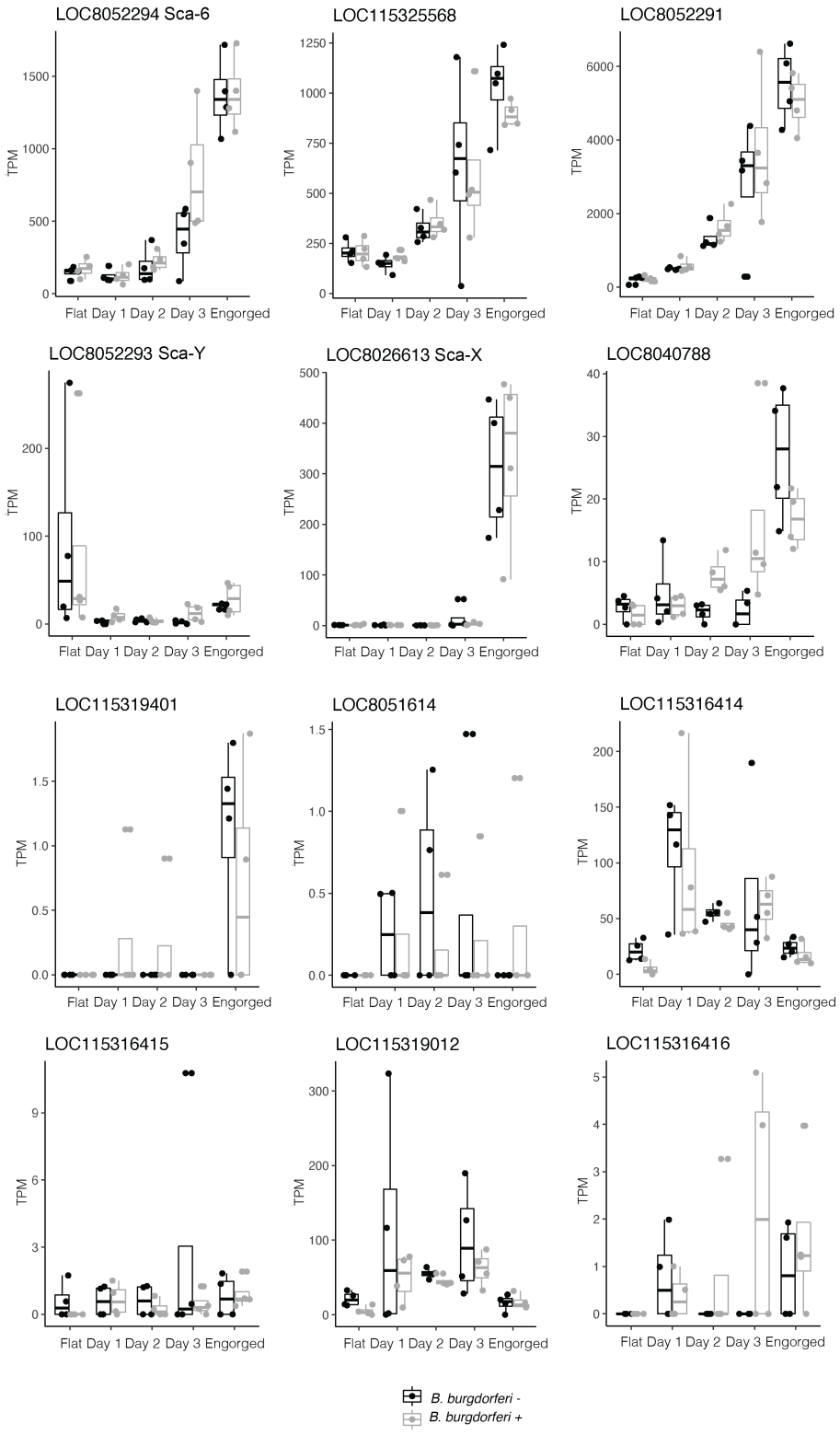

Figure 3.3. One scapularisin shows significant change in expression when comparing *B. burgdorferi* infected and uninfected nymphs. Expression of scapularisins in *B. burgdorferi* infected (grey) and uninfected (black) nymphs. Plots show transcripts per kilobase million (TPM) during each time point from the nymphal bloodmeal. Each replicate is composed of a

pool of five ticks (n = 3-4). Overall, there is not a significant difference in gene expression patterns between groups at any timepoint. The only exception is LOC8040788 expression at Day 2 is significantly higher in infected ticks compared to uninfected ticks (padj = 0.019).

feeding have identified that *Borrelia* numbers in the tick have a significant decrease detectable at 36 hours of feeding and these lower numbers persists throughout the rest of feeding[55]. This decrease is unlikely a reflection of transmitted *Borrelia*, as less than 0.1% of this tick-borne pathogen successfully enter the salivary glands where they can be transmitted through saliva[21]. If scapularisin expression is one method for ticks to limit the number of *Borrelia* they harbor, I hypothesized that scapularisin expression would further increase in *B. burgdorferi* infected nymphs. An RNA-seq experiment was conducted to evaluate gene expression changes of infected nymphs over the feeding time course. Scapularisin expression from *B. burgdorferi* infected ticks was compared to scapularisin expression from uninfected ticks using data from the previous RNA-seq experiment (**Fig 3.1, Fig 3.2**). This evaluation revealed only one scapularisin, LOC8040788, had a significant increase ($\log_2$ fold change = 1.78) in *B. burgdorferi* infected ticks at the Day 2 timepoint (padj = 0.019) and this increase in expression was also observed on Day 3 although the significant difference was not maintained (padj = 0.107) (**Fig 3.3**). Despite these differences, the overall expression trend for LOC8040788 in *B. burgdorferi* infected and uninfected ticks remained the same and trended upward over feeding, reaching a maximum at engorgement. This evidence suggests that LOC8040788 could increase expression in response to *B. burgdorferi* at time points associated with reduced *Borrelia* populations and this specific antimicrobial peptide-pathogen interaction may be worth further characterization.

The remaining 11 scapularisins did not have any significant differences in expression at any timepoints when comparing *B. burgdorferi* infection status (**Fig 3.3**), suggesting that scapularisin expression as a whole does not respond to this tick-borne pathogen. Assessment of

gene expression changes in the presence of other tick-borne pathogens and tick endosymbionts is warranted to fully evaluate the possibility of scapularisins limiting the proliferation of these microbes in the tick. Additional studies are required to develop an understanding of scapularisin activity during the late timepoints in tick feeding when expression reaches a maximum.

Scapularisins have a dual function as an agonist for MRGPRX2, a Mas-related G protein-coupled receptor

Defensins are well characterized as antimicrobial agents; however, there is a growing body of evidence demonstrating the ability of these AMPs to carry out alternative activities, such as stimulating tissue repair or immunomodulation[18,56]. A number of studies investigating alternative roles of defensin AMPs have focused on mammalian peptides and their role as mast cell activators[18]. Mast cells are sentinels in the skin and are activated upon recognizing stimuli, including pathogen associated molecular patterns (PAMPs)[57]. Mast cell activation causes these cells to degranulate and release preformed mediators that increase vascular permeability at the site of activation, increasing blood supply, and recruiting immune cells that promote wound healing (**Fig 3.4**)[33]. These are host-mediated processes that the tick may take advantage of during the blood feeding process[41]. One receptor that can initiate mast cell degranulation is MRGPRX2[39]. MRGPRX2 is known to interact with human defensins and is also stimulated by cationic peptides from wasp venom, such as mastoparan[18,58]. I hypothesized that scapularisins would serve as mast cell activators, specifically by interacting with MRGPRX2 on the surface of mast cells.

To assess whether Sca-6, Sca-X, and Sca-Y serve as ligands for MRGPRX2, I collaborated with Xintong Dong and James Limjunyawong from Xinzhong Dong's lab to carry out an *in vitro* assay using HEK 293 cells expressing MRGPRX2. Cells were treated with

increasing concentrations of each scapularisin and MRGPRX2 activation was determined by quantifying intracellular free calcium (**Fig 3.4**). All scapularisins were able to activate MRGPRX2. We determined the concentration which produces 50% of the maximal response (EC_{50}) for each AMP. Sca-6 had the highest EC_{50} at 4.4418 uM. The EC_{50} for Sca-X was the lowest at 0.9582 uM, and Sca-Y had an intermediate EC_{50} of 1.340 uM. These differences in EC_{50} correlate with the positive charge of each mature peptide: Sca-X and Sca-Y have a positive charge of 7.2 and each have a lower EC_{50} than Sca-6, which has a positive charge of 3.4.

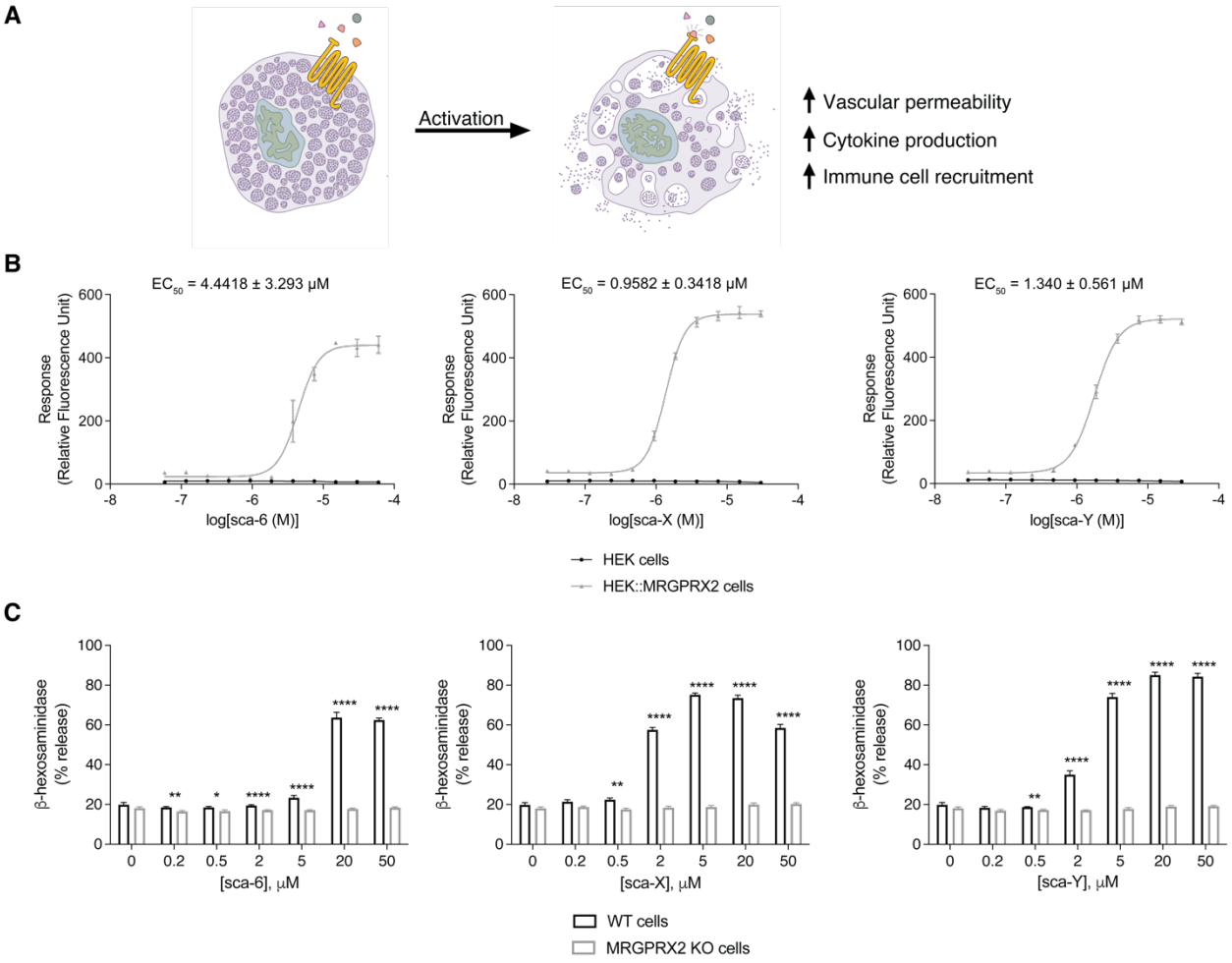

Figure 3.4. MRGPRX2 is necessary for scapularisin mediated mast cell degranulation. (A) A schematic showing MRGPRX2 (yellow) on the surface of mast cells. Upon binding a ligand, the cell is activated and degranulates, releasing a number of preformed mediators. Mast cell degranulation leads to increased vascular permeability, immune cell recruitment, and *de novo* cytokine production. (B) Dose-response curves of HEK293 cells expressing MRGPRX2. Response (Relative Fluorescence Unit) is a readout for intracellular free calcium after

stimulation by each scapularisin. The concentration which produces 50% of the maximal response (EC_{50}) is reported for each scapularisin. The mean response is plotted for each concentration tested and error bars represent standard error of the mean (SEM). (C) Each scapularisin was incubated with wild type (WT) or MRGPRX2 knockout (KO) mast cells (LAD2 cell line) at varying concentrations. Mast cell degranulation was assayed via β-hexosaminidase release and activity. β-hexosaminidase release increased in WT cells treated with increasing concentration of scapularisins. KO cells did not show an increase in β-hexosaminidase activity, indicating that MRGPRX2 is necessary for scapularisin mediated mast cell degranulation. Each bar represents the mean β-hexosaminidase release and error bars represent SEM.

Having identified these scapularisins as MRGPRX2 activators, we further tested our hypothesis that scapularisins activate mast cells by working with the LAD2 mast cell line. Both wild type and MRGPRX2 knockout LAD2 cell lines were incubated with increasing concentrations of each scapularisin (**Fig 3.4**). Mast cell degranulation was assayed via the release and activity of an enzyme found in mast cell granules, β-hexosaminidase[18]. We found that all three scapularisins tested led to a significant increase in β-hexosaminidase release from wild type cells when compared to the MRGPRX2 knockout (**Fig 3.4**). Consistent with the EC_{50} data (**Fig 3.4**), Sca-X and Sca-Y initiate degranulation at a lower concentration of peptide than Sca-6. Sca-X, the scapularisin with the lowest determined EC_{50}, initiated mast cell degranulation at a lower concentration than Sca-6 and Sca-Y. Sca-X and was the least effective AMP when tested in previous MIC and MBC assays (**Fig 2.3, Fig 2.4**). This data suggests that variation among scapularisins may support distinct functions during blood feeding.

Importantly, this mast cell experiment reveals an absence of β-hexosaminidase release among the MRGPRX2 knockout line when treated with Sca-6, Sca-X, and Sca-Y. This demonstrates that MRGPRX2 is necessary for scapularisin mediated mast cell degranulation (**Fig 3.4**). Scapularisin mediated activation of the innate immune system late during tick feeding may be one way ticks manipulate their host to enhance the tick's ability to blood-feed.

Ticks fed on mice with MRGPRX2 ortholog knocked out have a decrease in weight during blood feeding

Components of tick saliva mediate processes occurring at the bite site interface to promote successful tick feeding. Increasing scapularisin expression late in feeding and secreting these AMPs into the bite site may be one example of this phenomenon. Scapularisin mediated activation of MRGPRX2 could enhance the amount of blood at the bite site by inducing the release of histamine and heparin from mast cell granules, which promote blood flow and prevent blood clotting. I hypothesized that scapularisin mediated mast cell activation via MRGPRX2 provided a benefit to feeding *I. scapularis* nymphs. To test this hypothesis, I fed nymphs on wild type or *Mrgprb2* knock out (KO) mice for three days before forcibly removing nymphs. Mrgprb2 is the mouse ortholog to human MRGPRX2 and is present on murine mast cells[39]. After collecting nymphs from the feeding experiment, each tick was individually weighed, and the number of recovered ticks was used to calculate the percent of attached nymphs for each mouse (**Fig 3.5**). There was not a significant difference in percent attachment when comparing ticks fed on wild type and *Mrgprb2* KO mice (mouse n=4). When evaluating differences in weight, ticks fed on *Mrgprb2* KO mice had a highly significant decrease in weight when compared to ticks fed on wild type mice (wild type mean = 1.273 mg, *Mrgprb2* KO mean = 0.92 mg; wild type median = 0.95, *Mrgprb2* KO median = 0.80; p-value = $<2.2\text{x}10^{-6}$). (**Fig 3.5**). Statistical analysis of tick weights between groups was done using a Wilcoxon rank test for clustered data under the guidance of Dr. Karla Lindquist. By clustering during statistical analysis, I was able to account for a possible correlation between ticks fed on the same mouse. This highly significant difference between groups strongly suggests that the absence of *Mrgprb2* negatively impacts the ability of ticks to feed to a degree equal to those fed on wild type mice. This finding supports the

hypothesis that the scapularisin-MRGPRX2 interaction, or scapularisin-Mrgprb2 in mice, benefits tick blood feeding and is an area of research worthy of future exploration.

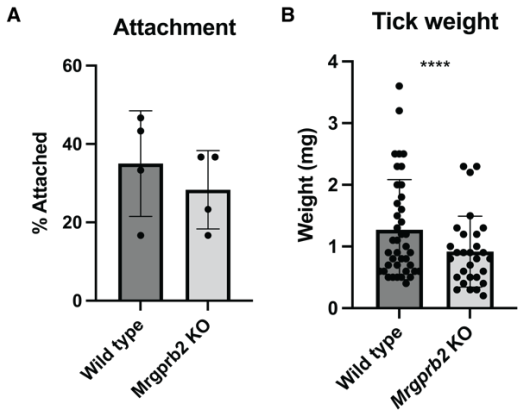

Figure 3.5. Ticks fed on *Mrgprb2* knockout mice have decreased weight. (A) *I. scapularis* nymphs were placed on wild type or *Mrgprb2* knockout (KO) mice and fed for three days before being forcibly removed. The percent of attached ticks was determined by comparing the number of ticks retrieved on day three to the total number of ticks applied on each mouse. No significant difference in attachment rate was determined (n=4). Bars represent the mean percent attached and errors bars are 1 standard deviation (SD). (B) Individual ticks were weighed after feeding on wild type or *Mrgprb2* KO mice for three days. Each dot represents an individual tick (wild type n = 40, *Mrgprb2* KO n = 29). Nymphs fed on *Mrgprb2* KO mice had statistically significant lower weights than ticks fed on wild type mice (wild type mean = 1.273 mg, *Mrgprb2* KO mean = 0.92 mg; wild type median = 0.95, *Mrgprb2* KO median = 0.80; p-value = $<2.2x10^{-6}$). Bars represent the mean weight and errors bars are 1 SD.

Discussion

Venomous tick saliva has known impacts on host physiology. Identification and characterization of specific components in this secretion is an active area of research which seeks to understand their molecular interactions between ticks and the organisms they encounter. Scapularisins secreted in *I. scapularis* venomous saliva execute both their canonical antimicrobial activity and a novel role as mast cell activating peptides via the Mas-related G protein-coupled receptor MRGPRX2. Given the antimicrobial function of scapularisins and the observed expression patterns, this work supports a model that scapularisins function during the early feeding and late feeding stages (**Fig 3.6**). The scapularisin gene expansion may benefit *I. scapularis* by allowing *I. scapularis* to increase the expression of AMPs from the defensin

family when they are needed during the late feeding stage[6,25,26]. This expansion of scapularisins could promote evolution of individual scapularisins to have specialized functions at different times during tick blood feeding, including MRGPRX2/ Mrgprb2 activation (**Fig 2.10**).

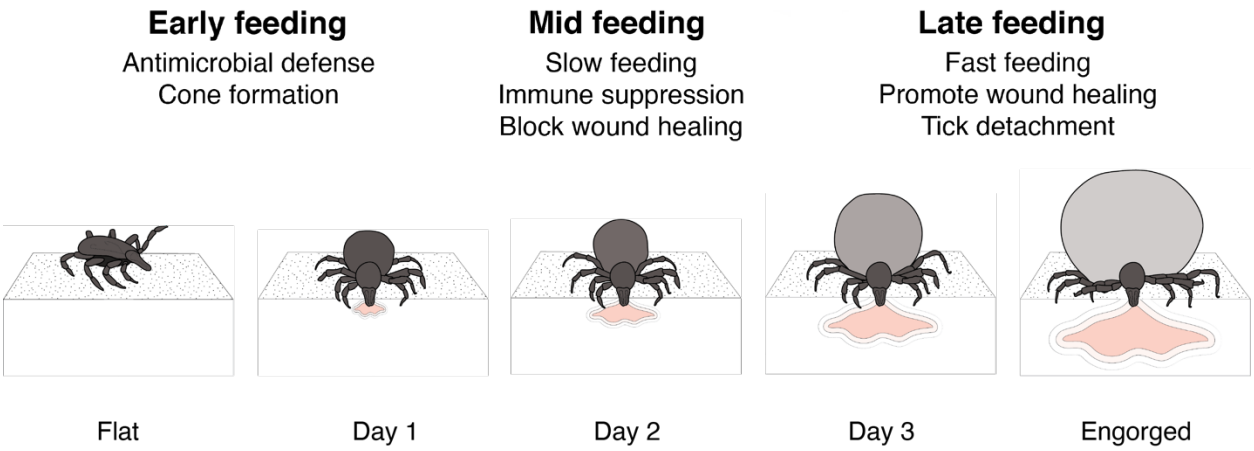

Figure 3.6. Scapularisins function as antimicrobials during early feeding stages and promote processes mediated by mast cells late in blood feeding. This model highlights a few of the processes taking place in the early, mid, and late feeding stages of *I. scapularis*. Antimicrobial activity against host skin commensals is important during early feeding when ticks are first encountering these microbes. Nymphs also create a cement cone, which is the site of venomous saliva injection into the host and uptake of a bloodmeal from the host. The mid feeding stage is associated with slow blood feeding by the tick and active suppression of the host's immune system, including the inhibition of wound healing processes. The late feeding stage is characterized by rapid engorgement of the tick, healing the wound at the bite site, and ultimately tick detachment. Scapularisins play a role in antimicrobial defense early in feeding. They also have a novel role late in feeding by activating mast cells to initiate processes that promote bringing blood to the bite site and possibly wound healing and tick detachment.

Exploring an alternative role for cationic AMPs was supported by existing studies in various fields of research, including studies of venom and peptides from venomous creatures[18,25]. Numerous studies have also looked at the effect of venom on mast cells, helping to strengthen the relationship between venomous peptides and mast cell activation[25,32,59]. Mast cell activation in response to parasites in skin is a well characterized phenomenon[17,41]. The research documented here ties these concepts together. Additionally, this study of defensins from the hard tick *I. scapularis* (Ixodidae) supports data from a study describing increasing defensin expression during the course of blood feeding for the soft tick *Ornithodoros moubata* (Argasidae)[54]. This

demonstrates that these well conserved AMPs have conserved expression patterns among tick families with lineages diverging 290±23 MYA and increases the evidence that these AMPs provide a benefit to ticks during blood feeding[60].

Data evaluating scapularisin gene expression in *B. burgdorferi* infected or uninfected ticks largely supports previous studies which suggest that scapularisins do not target the Lyme disease causing bacteria[5]. One possible exception to this is the scapularisin LOC8040788, which had a significant up-regulation in response to *B. burgdorferi* at a single timepoint associated with decreased *Borrelia* numbers in ticks[55]. That data point may encourage future characterization of the interaction between this AMP and *B. burgdorferi*. Future studies should similarly characterize how the presence of other tick-borne pathogens or endosymbionts affects tick AMP expression, including scapularisins.

Moving forward, studies of tick derived venomous peptides, specifically those that function as MRGPRX2 agonists, should explore the interaction between agonist and this receptor on mast cells. Different orthologs of MRGPRX2 have varying sensitivities to basic secretagogues, as evidenced when comparing the higher agonist concentrations required to activate murine Mrgprb2 in comparison to human MRGPRX2[36,61]. Rodents are common hosts to ticks during their early life stages and ticks may utilize the decreased sensitivity of Mrgprb2 to time activation of this receptor when it benefits them most at the late feeding stage[3]. Comparative studies evaluating the sensitivity to scapularisins by MRGPRX2 orthologs from various bloodmeal hosts, including incidental hosts like dogs and humans, may reveal an evolutionary preference for bloodmeal hosts with less sensitive pattern recognition receptors (PRRs) on mast cells. From the perspective of the vertebrate host, having a PRR on an immune

cell that quickly encounters and recognizes conserved venomous peptides is an advantage in the arms race between vertebrate host and hematophagous parasite.

The finding that nymphs feeding on *Mrgprb2* KO mice have decreased weight when compared to nymphs fed on wild type mice demonstrates that *I. scapularis* nymphs benefit from processes initiated by the activation of MRGPRX2/ Mrgprb2 receptors on mast cells. It is possible that ticks feeding on *Mrgprb2* KO mice feed more slowly and future studies should ask if this difference in weight is diminished if nymphs are fed to engorgement on *Mrgprb2* KO mice. A better characterization of these temporal effects on feeding ticks will create a more complete understanding of the scapularisin-*MRGPRX2* interaction *in vivo*. Furthermore, scapularisins should be examined for their ability to lyse red blood cells as this activity is seen in defensins from *I. ricinus* and may explain some of the blood feeding defects identified in this work[19].

Understanding how ticks mediate interactions with other organisms enables scientists to create tools which directly benefit human health and the well-being of numerous animals negatively affected by hematophagous parasites. Characterization of these molecular interactions gives us the ability to learn more human biology as we expose how ticks manipulate vertebrate hosts while simultaneously elucidating fundamental tick biology. Given that immune systems are a common target of venom, these studies may reveal new approaches to activate or inhibit vertebrate immune systems to combat diseases.

Methods

Animals

Animal experiments were conducted in accordance with the approval of the Institutional Animal Care and Use Committee (IACUC) at UCSF, which maintains full AAALAC accreditation.

C3H/HeJ mice (aged 4-6 weeks) from Jackson Laboratories were used for the tick-feeding experiments unless noted otherwise. UCSF Laboratory Animal Resource Center (LARC) or trained Chou lab personnel performed daily health checks for mice.

Tick feeding

Certified pathogen free *I. scapularis* nymphs and adults were purchased from the tick lab at Oklahoma State University (OSU). Ticks were maintained in a sealed incubator with a cycle of 16 hours light/ 8 hours dark. The incubator was kept at 22 °C and ticks were stored in glass chambers with a relative humidity of 95% (saturated solution of potassium nitrate). Animal experiments were conducted in accordance with the approval of the Institutional Animal Care and Use Committee (IACUC) at UCSF. Ticks were fed on C3H/HeJ mice obtained from Jackson Laboratories unless otherwise noted. 30 ≤ or ≤10 adult female ticks were placed on mice anesthetized with ketamine/ xylazine and mice were immediately placed in a warm, humid cage. Ticks were pulled from anesthetized mice at various points during feeding or collected from mouse cages upon engorgement and detachment from mice.

Synthesized peptides

Sequences for peptide synthesis were obtained from the *I. scapularis* ISE6 cell line genome (GenBank accession GCA_002892825.2): Sca-6 XP_029842170.1, Sca-X XP_002404709.2, Sca-Y XP_029839602.1. Only the amino acids following the furin cleavage motif (RVRR) were chemically synthesized in order to generate mature peptides. Peptides were synthesized with 95% purity using standard procedures (Peptide 2.0). Lyophilized peptides were solubilized in low salt buffer (100 nM NaCl and 20 mM HEPES, pH 7).

RNA extraction from ticks

All ticks were surface sterilized in 1% bleach for 5 minutes before being rinsed in nuclease free water. Ticks were then pooled into a 2 mL screw cap tube containing 500 uL of Trizol reagent (Invitrogen 15596026) and 4-6 1.4 mM ceramic beads (Omni International 19-645). Each tube was placed on a bead beater and shaken at 4000 RPM for 20 seconds and placed on ice for a minimum of 30 seconds. This process was repeated twice more for a total of 60 seconds of bead beating. RNA was extracted following the Trizol reagent standard procedure. Extracted RNA was treated with TURBO DNase (Thermo Fisher AM2238) and quantified using Qubit RNA HS assay (Invitrogen Q32852).

RNA-sequencing library preparation and sequencing

Either 10 ng, 100 ng, or 250 ng of total RNA extracted from pools of 5 ticks at the appropriate time point/ infection status was used as input for NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs E7490S) and NEBNext Ultra II directional RNA Library Prep Kit for Illumina (New England Biolabs E7760S) using BioRad CFX Connect (1855201). SYBR green was added to amplification step to determine the appropriate number of cycles. RNA-sequencing libraries were run on an Illumina NovaSeq SP with paired-end 150 base-pair reads.

Data analysis for RNA-seq

Paired end fastq files were trimmed and quality filtered using cutadapt version 2.8[62]. Reads were filtered to those at least 30 bases long with a minimum 3' quality of 10. (flags -m 30 -q 10). Quality-filtered reads were then pseudo-aligned to the *Ixodes scapularis* reference genome version GCF_002892825.2_ISE6_asm2.2_deduplicated using kallisto version 0.46.1 with 100

bootstraps (flag -b 100)[63]. Differential expression analysis was performed using DESeq2 version 1.34[64]. R notebook with details can be found at github.com/callamartyn/iscap_rnaseq. Genes were filtered to those with at least 5 counts across the dataset and differential expression testing was performed using DESeq2 defaults. Differentially expressed genes were determined by modeling gene expression as a function time using the flat time point for comparison or controlling for *Borrelia* infection status.

EC50 determination

Human embryonic kidney (HEK) 293 cells stably expressing Gα15 and MRGPRX2 were plated at 5 × 104 cells per well in black 96-well plates and incubated overnight. The following day, media was removed and replaced with 100 µl FLIPR Calcium 5 dye (Molecular Devices), diluted in Hank's balanced salt solution (HBSS) with 20 mM HEPES, pH 7.4. Cells were then incubated at 37 °C for 60 min and equilibrated at room temperature for 15 min before imaging. Imaging was performed in Flexstation 3 device with an integrated pipetting system (Molecular Devices) at 485/525 nm (excitation/emission). Emission were recorded every 2 seconds for 180 seconds, with 50 µl of serial dilution of scapularisins (3X final concentration) added at 20 seconds. Responses were defined as maximal signal minus minimal signal of each well. Experiments were repeated 3 times independently, each in triplicate. Concentration-Response curves were generated and EC50 values were calculated using GraphPad Prism software (GraphPad Inc.).

Mast cell degranulation assay

The human WT LAD2 (Laboratory of Allergic Disease-2) cells were kindly provided by Dr. Arnold Kirshenbaum and Dr. Dean Metcalfe (National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, MD). MRGPRX2 knockout (KO) LAD2 cells were generated using CRISPR/Cas9 technology as previously described[65]. The cells were cultured in StemPro-34 culture media (Gibco) containing the StemPro-34 nutrient supplement, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 100 ng/ml recombinant human stem cell factor (Peprotech). For degranulation assay, WT and MRGPRX2 KO LAD2 cells (2×10^4 cells/well) were seeded and stimulated with different concentration of scapularisins for 30 min at 37 °C/5% CO_2. Then, cells were pelleted, supernatants were collected, and cells were solubilized with 0.1% Triton-X100. The β-hexosaminidase in the supernatants and cell lysates were quantified by hydrolysis of p-nitrophenyl *N*-acetyl-β-D-glucosamide (Sigma-Aldrich) in 0.1 M sodium citrate buffer (pH 4.5) for 90 min at 37 °C. The reaction was stopped by adding 0.4 M glycine solution (pH 10.7) and the absorbance was read at 405 nm with reference wavelength at 570 nm. The percentage of β-hexosaminidase release was calculated as a percentage of enzyme activities present in the supernatants comparing to total activities in both supernatants and cell lysates.

Tick feeding on *Mrgprb2* knockout mice

I. scapularis nymphs from OSU were fed on wild type C57BL/6J mice (Jackson Laboratories) or *Mrgprb2* knockout C57CL/6J mice generously provided by Dr. Xinzhong Dong (Johns Hopkins University)[39]. Ticks were forcibly removed from mice on the third day of feeding and weighed using an analytical balance. Percent attachment was determined by comparing the number of

live, recovered ticks to the total number of ticks applied to each mouse and significance was examined using a t-test in GraphPad Prism software (GraphPad Inc.). Statistical analysis of tick weights between groups was done in R using clusrank, a Wilcoxon rank test for clustered data.

Chapter 4: Exploratory experiments with *Ixodes* ticks and *Borrelia burgdorferi*

Introduction

As a member of a new lab, there were many directions that my graduate work could explore. I was the first graduate student in the lab to take on a project focusing on tick biology. The early years of my graduate work consisted of following my curiosity and investigating new research projects. A consistent theme of these new research projects was investigating how as a single organism, a tick could manage many interactions with organisms from other kingdoms.

The work summarized in chapters two and three focused on tick-microbe and tick-host interactions, comprising the majority of my scientific research over the last five years. However, there were a few promising exploratory experiments that may serve as a starting point for future projects. Two such project directions are summarized below. These sections include a brief introduction to the research question, an explanation of data, and suggestions for how to modify previously done experiments or potential next steps.

Assessing physical parameters of *B. burgdorferi* dissemination in *I. scapularis*

The ability of *Ixodes* ticks to acquire, harbor, and transmit *Borrelia* species is well documented. However, the molecular processes inside the tick that enable vector competency are poorly understood. One aspect of this tick-microbe interaction that needs further exploration is how *B. burgdorferi* exits the midgut and enters the hemocoel, a body cavity containing circulatory fluid and hemocytes (immune cells), and eventually migrates to the salivary glands where it is then transmitted into the host animal via tick saliva. This dissemination step is a bottleneck in transmission as less than 0.1% of *B. burgdorferi* successfully exit the midgut and enter the salivary glands[21]. An open question regarding *B. burgdorferi* dissemination inside tick asks if this is an active bacterial process or a passive dissemination mediated by the influx of

blood and return of water to the host via tick saliva as ticks compact nutrients from the bloodmeal[66]. I chose to investigate this physical aspect of *B. burgdorferi* dissemination.

The method for addressing physical parameters of *B. burgdorferi* dissemination was adapted from a model evaluating *Rickettsia felis* transmission mechanics in fleas[67]. I modified the blood feeding procedure by first injecting *I. scapularis* females with either 1.0 μm or 0.1 μm sized fluorescent beads (Sigma L2778 and L9904). These sizes were chosen based on product availability and the dimensions of *B. burgdorferi*'s unique cell morphology (approximately 0.3 μm by 10-20 μm)[68]. Nymph and adult *I. scapularis* were microinjected with varying numbers of each size of fluorescent beads, ranging from a total concentration of 10^{10} to 10^{4}, and evaluated after 24 hours to assess lethality of bead injections. After 24 hours, all nymphs were deceased, and all adults survived. Moving forward, adult female ticks were injected with a final concentration of ~4.5 x 10^{5} 1 μm beads. The experimental approach was intended as follows: 24 hours after bead microinjection, five females were placed on a mouse and allowed to feed until engorgement. At engorgement, salivary glands were to be dissected from each tick and inspected for fluorescent beads. After 14 days of blood feeding, only one female reached engorgement and detached. The remaining 4 ticks were forcibly removed on the 15th day of feeding. This incomplete feeding may be due to their preference for feeding on larger animals during this final lifestage[3]. Our IACUC protocol limited me to feeding adult ticks on mice, but future iterations of this experiment may benefit from feeding on larger mammals, like rabbits.

Salivary glands were imaged using spinning disk confocal microscopy and assistance from Kari Herrington at UCSF's Nikon Imaging Center. Beads appear to be present in the dissected salivary glands (**Fig 4.1**), suggesting that dissemination of *B. burgdorferi* may be a passive process that occurs as ticks expel water from their bloodmeal back into the host. Future

studies should assess this concept using a more rigorous approach. One such improvement could be injecting fluorescent beads that more closely resemble spirochete cell morphology. Additionally, more tissues should be examined for the presence of fluorescent beads. These include the midgut, hemolymph (including hemocytes capable of phagocytosing beads), and saliva. Thoroughly investigating the passive dissemination of particles through the tick during blood feeding may reveal that this is truly a passive process and could explain why so few *B. burgdorferi* successfully enter the salivary glands.

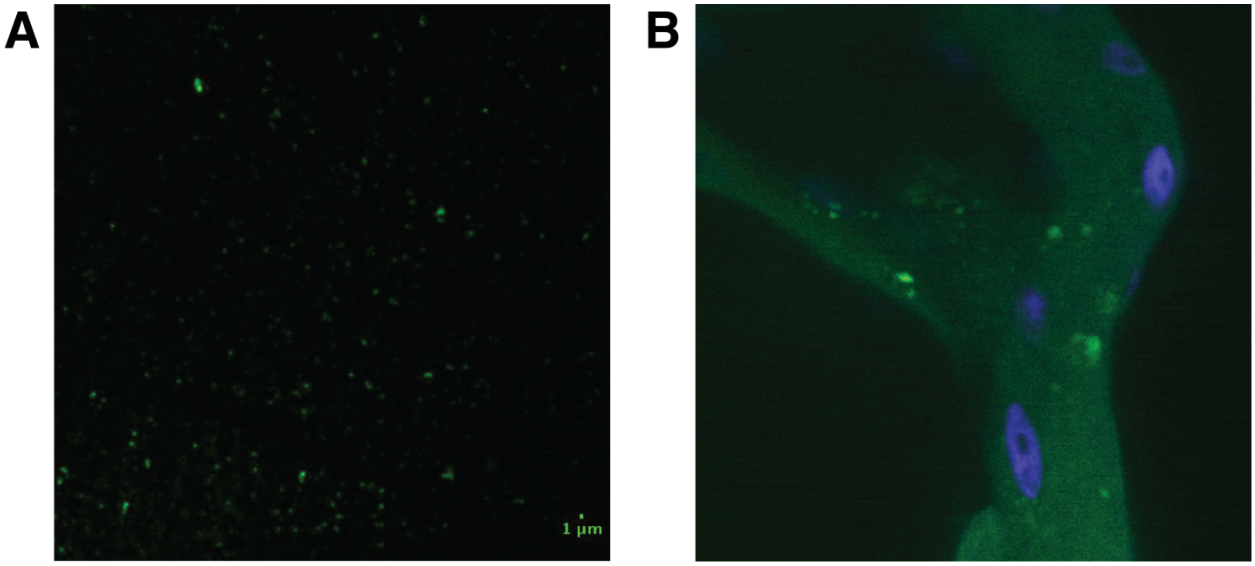

Figure 4.1. Fluorescent beads are visible in tick salivary glands after tick feeding. (A) Control images of 1 µm beads using confocal microscopy. (B) Merged image showing beads (green) in dissected salivary glands. Nuclei are stained with DAPI (purple).

Investigating the borreliacidal activity of blood from the western fence lizard (*Sceloporus occidentalis*)

In 1998, Lane and Quistad published work describing borreliacidal activity of blood from the Western fence lizard, *Sceloporus occidentalis*[69]. This lizard inhabits regions west of the Rocky Mountains and is a common host for larval and nymphal *Ixodes pacificus*, the Western blacklegged tick[70]. Both *I. pacificus* and *I. scapularis* are competent vectors for *B. burgdorferi*

and these tick species have a close evolutionary relationship[71]. Despite the fact that both tick species can transmit the Lyme disease bacterium and *B. burgdorferi* is present on both coasts of the United States, prevalence of Lyme disease is lower on the West Coast[3]. The observation that Western fence lizards were not competent reservoirs for *B. burgdorferi* due to the borreliacidal agent in their blood was proposed to have a role in this decreased disease prevalence[69]. Through this initial work, it was determined that the borreliacidal agent is likely a protein larger than 30 kDa[69]. Additional work by Kuo et al. determined that the borreliacidal protein is part of the alternative complement pathway[72]. Data from this same study determined that the mammalian alternative complement pathway is not as effective at killing *B. burgdorferi*, suggesting an evolutionary divergence between the alternative complement pathway in reptiles and mammals[72]. Details about these differences have yet to be determined. The work in this section attempted to validate the findings summarized above with the intention of moving forward to identify the component of the *S. occidentalis* alternative complement pathway with borreliacidal activity.

Due to limitations in our animal protocol, breeding of *S. occidentalis* to collect serum was not an option. I was fortunate enough to be in contact with Dr. Sima Bouzid who provided me with blood samples from Western fence lizards collected in California during her graduate work in 2018[73]. Upon receipt of these samples, they were centrifuged, and serum was stored at -80°C. The *B. burgdorferi* B31 strain was used in viability experiments following established protocols which accounted for motile and non-motile *Borrelia* under a microscope after incubation with sera or controls for one hour[69]. Data from the experiment with *B. burgdorferi* B31 demonstrated a decreased level of *B. burgdorferi* mortality compared to previous studies, although borreliacidal activity was still observed (**Fig 4.2**). This discrepancy may be caused by the low

number of replicates (n=2) or the use the B31 strain instead of the CA4 strain. The *B. burgdorferi* CA4 stain was used in the initial borreliacidal activity experiments with *S. occidentalis* serum and is likely a better choice for these experiments as this strain of bacteria originated in California and is more likely to have evolved with the Western fence lizard[69]. Future studies would likely benefit from an increased number of replicates and use of *B. burgdorferi* strains commonly found in or previously collected from *I. pacificus* ticks.

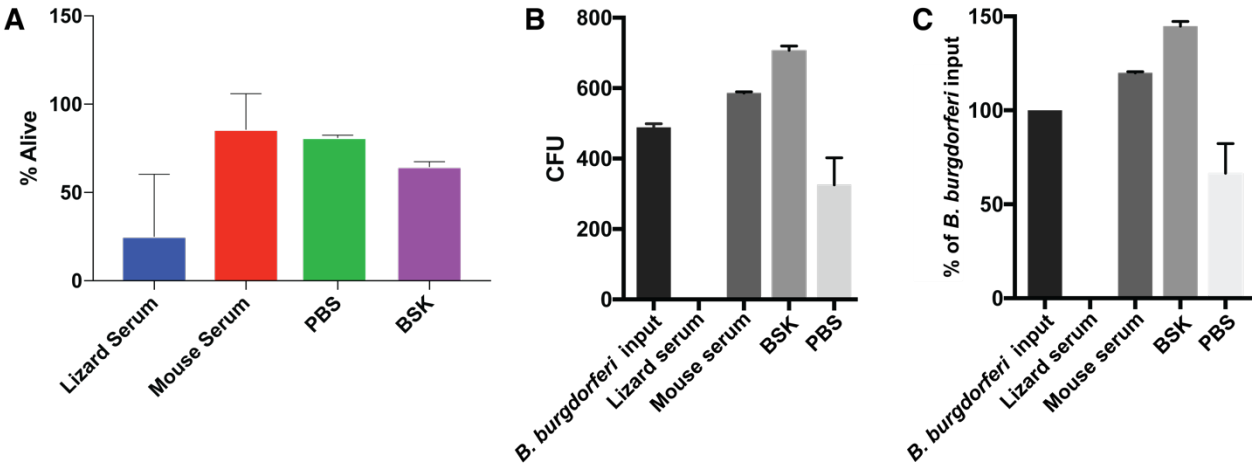

Figure 4.2. Confirmation of borreliacidal activity from *Sceloporus occidentalis* serum. (A) Assessment of viability (% alive) of *B. burgdorferi* B31 after a 1 hour incubation with serum from *S. occidentalis* (western fence lizard), *M. musculus* (mouse), and controls PBS and BSK II (Barbour-Stoenner-Kelly II medium for growth of *B. burgdorferi* in liquid culture)[74]. Viability was assessed by viewing 100 spirochetes under a microscope and counting motile organisms[69]. Lizard serum was not completely borreliacidal after 1 hour of incubation (n=2). (B) *B. burgdorferi* was plated using BSK 1.5 media after incubation with sera or controls. A total number of 500 *B. burgdorferi* cells was used in each incubation and *B. burgdorferi* input refers to bacteria that received no treatment. After 4 hours of incubation, complete borreliacidal activity in the lizard serum samples prevented the any colony forming units (CFU) from being observed (n = 2). (C) Data from B was plotted by normalizing CFU from each treatment to the average number of CFU from *B. burgdorferi* input (489).

In additional to assessing mortality via motility of organisms under the microscope, *B. burgdorferi* were plated using established procedures after 4 hours of incubation with sera to determine colony forming units (CFU)[75]. This approach to determine viability of *Borrelia* after incubation with antimicrobial agents is in line with conventional assays to determine bactericidal

activity[50]. These experiments showed borreliacidal activity after 4 hours of incubation in western fence lizard serum (**Fig 4.2**), confirming the presence of a borreliacidal agent in *S. occidentalis* serum.

Having reproduced findings that support the borreliacidal activity of *S. occidentalis* serum, I was interested in moving forward with characterizing the >30 kDa protein present in western fence lizard serum[69,72]. Advances in sequencing have enabled the generation of a transcriptome for *S. occidentalis*, which could facilitate efforts to identify the borreliacidal agent using proteomics tools, including mass spectrometry[76]. However, advancing this project in our laboratory was difficult due to constraints involving animal care requirements for *S. occidentalis*. Either laboratory rearing or field collection of western fence lizards would have been required to collect addition serum necessary for proteomics. Future explorations of this work need to ensure access to serum samples from *S. occidentalis*, which may be possible by attaining these organisms from a colony maintained at Oklahoma State University[76]. Additional research in this subject area is valuable because it will allow a comparative examination of the alternative complement pathway in reptiles and mammals. Given that other reptiles have demonstrated borreliacidal activity, learning more about how the reptilian alternative complement pathway combats these tick-borne pathogens creates new opportunities to combat *Borrelia* infections.

References

1. Tonk, M. *et al.* Defensins from the tick Ixodes scapularis are effective against phytopathogenic fungi and the human bacterial pathogen Listeria grayi. *Parasite Vector* 7, 554 (2014).

2. Vechtova, P. *et al.* Catalogue of stage-specific transcripts in Ixodes ricinus and their potential functions during the tick life-cycle. *Parasite Vector* 13, 311 (2020).

3. Kurokawa, C. *et al.* Interactions between Borrelia burgdorferi and ticks. *Nat Rev Microbiol* 18, 587–600 (2020).

4. Bryant, W. B. & Michel, K. Blood feeding induces hemocyte proliferation and activation in the African malaria mosquito, Anopheles gambiae Giles. *J Exp Biol* 217, 1238–1245 (2013).

5. Sonenshine, D., E. Molecular characterization and related aspects of the innate immune response in ticks. *Front Biosci* Volume, 7046 (2008).

6. Wang, Y. & Zhu, S. The defensin gene family expansion in the tick Ixodes scapularis. *Dev Comp Immunol* 35, 1128–1134 (2011).

7. Chávez, A. S. O., Shaw, D. K., Munderloh, U. G. & Pedra, J. H. F. Tick Humoral Responses: Marching to the Beat of a Different Drummer. *Front Microbiol* 8, 223 (2017).

8. Shaw, D. K. *et al.* Infection-derived lipids elicit an immune deficiency circuit in arthropods. *Nat Commun* 8, 14401 (2017).

9. Tsuzuki, S. *et al.* Drosophila growth-blocking peptide-like factor mediates acute immune reactions during infectious and non-infectious stress. *Sci Rep-uk* 2, 210 (2012).

10. Wang, G. Human Antimicrobial Peptides and Proteins. *Pharm* 7, 545–594 (2014).

11. Mandal, S. D. *et al.* Antimicrobial Peptides: Novel Source and Biological Function With a Special Focus on Entomopathogenic Nematode/Bacterium Symbiotic Complex. *Front Microbiol* 12, 555022 (2021).

12. Broderick, N. A. Friend, foe or food? Recognition and the role of antimicrobial peptides in gut immunity and Drosophila–microbe interactions. *Philosophical Transactions Royal Soc B Biological Sci* 371, 20150295 (2016).

13. Shafee, T. M. A., Lay, F. T., Phan, T. K., Anderson, M. A. & Hulett, M. D. Convergent evolution of defensin sequence, structure and function. *Cell Mol Life Sci* 74, 663–682 (2017).

14. Hajdušek, O. *et al.* Interaction of the tick immune system with transmitted pathogens. *Front Cell Infect Mi* 3, 26 (2013).

15. Johns, R., Sonenshine, D. E. & Hynes, W. L. Identification of a defensin from the hemolymph of the American dog tick, Dermacentor variabilis. *Insect Biochem Molec* 31, 857–865 (2001).

16. Huan, Y., Kong, Q., Mou, H. & Yi, H. Antimicrobial Peptides: Classification, Design, Application and Research Progress in Multiple Fields. *Front Microbiol* 11, 582779 (2020).

17. Quan, P. L., Sabaté-Brescó, M., Guo, Y., Martín, M. & Gastaminza, G. The Multifaceted Mas-Related G Protein-Coupled Receptor Member X2 in Allergic Diseases and Beyond. *Int J Mol Sci* 22, 4421 (2021).

18. Arifuzzaman, M. *et al.* MRGPR-mediated activation of local mast cells clears cutaneous bacterial infection and protects against reinfection. *Sci Adv* 5, eaav0216 (2019).

19. Chrudimská, T., Slaninová, J., Rudenko, N., Růžek, D. & Grubhoffer, L. Functional characterization of two defensin isoforms of the hard tick Ixodes ricinus. *Parasite Vector* 4, 63–63 (2011).

20. Hardy, J. L., Houk, E. J., Kramer, L. D. & Reeves, W. C. Intrinsic Factors Affecting Vector Competence of Mosquitoes for Arboviruses. *Annu Rev Entomol* 28, 229–262 (1983).

21. Dunham-Ems, S. M. *et al.* Live imaging reveals a biphasic mode of dissemination of Borrelia burgdorferi within ticks. *J Clin Invest* 119, 3652–3665 (2009).

22. Neelakanta, G., Sultana, H., Fish, D., Anderson, J. F. & Fikrig, E. Anaplasma phagocytophilum induces Ixodes scapularis ticks to express an antifreeze glycoprotein gene that enhances their survival in the cold. *J Clin Invest* 120, 3179–3190 (2010).

23. Eyerich, S., Eyerich, K., Traidl-Hoffmann, C. & Biedermann, T. Cutaneous Barriers and Skin Immunity: Differentiating A Connected Network. *Trends Immunol* 39, 315–327 (2018).

24. Hayes, B. M. *et al.* Ticks Resist Skin Commensals with Immune Factor of Bacterial Origin. *Cell* 183, 1562-1571.e12 (2020).

25. Cabezas-Cruz, A. & Valdés, J. J. Are ticks venomous animals? *Front Zool* 11, 47–47 (2014).

26. Fry, B. G. *et al.* The Toxicogenomic Multiverse: Convergent Recruitment of Proteins Into Animal Venoms. *Annu Rev Genom Hum G* 10, 483–511 (2009).

27. Ribeiro, J. M. C. & Mans, B. J. TickSialoFam (TSFam): A Database That Helps to Classify Tick Salivary Proteins, a Review on Tick Salivary Protein Function and Evolution, With Considerations on the Tick Sialome Switching Phenomenon. *Front Cell Infect Mi* 10, 374 (2020).

28. Ali, A. *et al.* Host Immune Responses to Salivary Components - A Critical Facet of Tick-Host Interactions. *Front Cell Infect Mi* 12, 809052 (2022).

29. Anguita, J. *et al.* Salp15, an Ixodes scapularis Salivary Protein, Inhibits CD4+ T Cell Activation. *Immunity* 16, 849–859 (2002).

30. Kim, T. K. *et al.* Ixodes scapularis Tick Saliva Proteins Sequentially Secreted Every 24 h during Blood Feeding. *Plos Neglect Trop D* 10, e0004323 (2016).

31. Scholl, D. C. *et al.* Immunomodulatory effects of tick saliva on dermal cells exposed to Borrelia burgdorferi, the agent of Lyme disease. *Parasite Vector* 9, 394 (2016).

32. HIRAI, Y. *et al.* A New Mast Cell Degranulating Peptide “Mastoparan” in the Venom of Vespula lewisii. *Chem Pharm Bulletin* 27, 1942–1944 (1979).

33. Abraham, S. N. & John, A. L. St. Mast cell-orchestrated immunity to pathogens. *Nat Rev Immunol* 10, 440–452 (2010).

34. Kroeze, W. K. *et al.* PRESTO-TANGO: an open-source resource for interrogation of the druggable human GPCR-ome. *Nat Struct Mol Biol* 22, 362–369 (2015).

35. Dong, X., Han, S., Zylka, M. J., Simon, M. I. & Anderson, D. J. A Diverse Family of GPCRs Expressed in Specific Subsets of Nociceptive Sensory Neurons. *Cell* 106, 619–632 (2001).

36. Subramanian, H., Gupta, K. & Ali, H. Roles of Mas-related G protein–coupled receptor X2 on mast cell–mediated host defense, pseudoallergic drug reactions, and chronic inflammatory diseases. *J Allergy Clin Immun* 138, 700–710 (2016).

37. Wedi, B., Gehring, M. & Kapp, A. The pseudoallergen receptor MRGPRX2 on peripheral blood basophils and eosinophils: Expression and function. *Allergy* 75, 2229–2242 (2020).

38. Porebski, G., Kwiecien, K., Pawica, M. & Kwitniewski, M. Mas-Related G Protein-Coupled Receptor-X2 (MRGPRX2) in Drug Hypersensitivity Reactions. *Front Immunol* 9, 3027 (2018).

39. McNeil, B. D. *et al.* Identification of a mast-cell-specific receptor crucial for pseudo-allergic drug reactions. *Nature* 519, 237–241 (2015).

40. Palomäki, V. A. B. & Laitinen, J. T. The basic secretagogue compound 48/80 activates G proteins indirectly via stimulation of phospholipase D–lysophosphatidic acid receptor axis and 5-HT1A receptors in rat brain sections. *Brit J Pharmacol* 147, 596–606 (2006).

41. Mukai, K., Tsai, M., Starkl, P., Marichal, T. & Galli, S. J. IgE and mast cells in host defense against parasites and venoms. *Semin Immunopathol* 38, 581–603 (2016).

42. Meiners, J. *et al.* IL-33 facilitates rapid expulsion of the parasitic nematode Strongyloides ratti from the intestine via ILC2- and IL-9-driven mast cell activation. *Plos Pathog* 16, e1009121 (2020).

43. Nuss, A., Sharma, A. & Gulia-Nuss, M. Genetic Manipulation of Ticks: A Paradigm Shift in Tick and Tick-Borne Diseases Research. *Front Cell Infect Mi* 11, 678037 (2021).

44. Gulia-Nuss, M. *et al.* Genomic insights into the Ixodes scapularis tick vector of Lyme disease. *Nat Commun* 7, 10507 (2016).

45. Bulet, P., Hetru, C., Dimarcq, J.-L. & Hoffmann, D. Antimicrobial peptides in insects; structure and function. *Dev Comp Immunol* 23, 329–344 (1999).

46. Miller, J. R. *et al.* A draft genome sequence for the Ixodes scapularis cell line, ISE6. *F1000research* 7, 297 (2018).

47. Zhang, L. & Gallo, R. L. Antimicrobial peptides. *Curr Biol* 26, R14–R19 (2016).

48. Teufel, F. *et al.* SignalP 6.0 predicts all five types of signal peptides using protein language models. *Nat Biotechnol* 1–3 (2022) doi:10.1038/s41587-021-01156-3.

49. Fischer, A. & Rausell, A. Primary immunodeficiencies suggest redundancy within the human immune system. *Sci Immunol* 1, (2016).

50. Wiegand, I., Hilpert, K. & Hancock, R. E. W. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat Protoc* 3, 163–175 (2008).

51. Mueller, J. H. & Hinton, J. A Protein-Free Medium for Primary Isolation of the Gonococcus and Meningococcus. *P Soc Exp Biol Med* 48, 330–333 (1941).

52. Acuff, N. V., LaGatta, M., Nagy, T. & Watford, W. T. Severe Dermatitis Associated with Spontaneous Staphylococcus xylosus Infection in Rag-/-Tpl2-/- Mice. *Comparative Med* 67, 344–349 (2017).

53. Parlet, C. P., Brown, M. M. & Horswill, A. R. Commensal Staphylococci Influence Staphylococcus aureus Skin Colonization and Disease. *Trends Microbiol* 27, 497–507 (2019).

54. Nakajima, Y., Naters-Yasui, A. V. D. G. van, Taylor, D. & Yamakawa, M. Antibacterial peptide defensin is involved in midgut immunity of the soft tick, Ornithodoros moubata. *Insect Mol Biol* 11, 611–618 (2002).

55. Wilhelmsson, P. *et al.* Prevalence, Diversity, and Load of Borrelia species in Ticks That Have Fed on Humans in Regions of Sweden and Åland Islands, Finland with Different Lyme Borreliosis Incidences. *Plos One* 8, e81433 (2013).

56. Lehrer, R. I., Bevins, C. L. & Ganz, T. Mucosal Immunology (Third Edition). *Part Mucosal Immune Syst Sect Amucosal Barrier Dev Physiology Mucosal Déf Sect Mucosal Barrier Dev Physiology Mucosal Déf* 95–110 (2005) doi:10.1016/b978-012491543-5/50010-3.

57. John, A. L. St. & Abraham, S. N. Innate Immunity and Its Regulation by Mast Cells. *J Immunol* 190, 4458–4463 (2013).

58. Subramanian, H. *et al.* β-Defensins Activate Human Mast Cells via Mas-Related Gene X2. *J Immunol* 191, 345–352 (2013).

59. Metz, M. *et al.* Mast Cells Can Enhance Resistance to Snake and Honeybee Venoms. *Science* 313, 526–530 (2006).

60. Mans, B. J., Klerk, D. de, Pienaar, R., Castro, M. H. de & Latif, A. A. The Mitochondrial Genomes of Nuttalliella namaqua (Ixodoidea: Nuttalliellidae) and Argas africolumbae (Ixodoidae: Argasidae): Estimation of Divergence Dates for the Major Tick Lineages and Reconstruction of Ancestral Blood-Feeding Characters. *Plos One* 7, e49461 (2012).

61. Hamamura-Yasuno, E., Iguchi, T., Kumagai, K., Tsuchiya, Y. & Mori, K. Identification of the dog orthologue of human MAS-related G protein coupled receptor X2 (MRGPRX2) essential for drug-induced pseudo-allergic reactions. *Sci Rep-uk* 10, 16146 (2020).

62. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *Embnet J* 17, 10–12 (2011).

63. Bray, N. L., Pimentel, H., Melsted, P. & Pachter, L. Near-optimal probabilistic RNA-seq quantification. *Nat Biotechnol* 34, 525–527 (2016).

64. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15, 550 (2014).

65. Shtessel, M. *et al.* MRGPRX2 Activation Causes Increased Skin Reactivity in Chronic Spontaneous Urticaria Patients. *J Invest Dermatol* 141, 678-681.e2 (2020).

66. Kazimírová, M. & Štibrániová, I. Tick salivary compounds: their role in modulation of host defences and pathogen transmission. *Front Cell Infect Mi* 3, 43 (2013).

67. Brown, L. D., Banajee, K. H., Foil, L. D. & Macaluso, K. R. Transmission mechanisms of an emerging insect-borne rickettsial pathogen. *Parasite Vector* 9, 237 (2016).

68. Hyde, J. A. Borrelia burgdorferi Keeps Moving and Carries on: A Review of Borrelial Dissemination and Invasion. *Front Immunol* 8, 114 (2017).

69. Lane, R. S. & Quistad, G. B. Borreliacidal factor in the blood of the western fence lizard (Sceloporus occidentalis). *J Parasitol* 84, 29–34 (1998).

70. Ring, K. *et al.* Host blood meal identity modifies vector gene expression and competency. *Mol Ecol* 31, 2698–2711 (2022).

71. Charrier, N. P. *et al.* A transcriptome-based phylogenetic study of hard ticks (Ixodidae). *Sci Rep-uk* 9, 12923 (2019).

72. Kuo, M. M., Lane, R. S. & Giclas, P. C. A COMPARATIVE STUDY OF MAMMALIAN AND REPTILIAN ALTERNATIVE PATHWAY OF COMPLEMENT-MEDIATED KILLING OF THE LYME DISEASE SPIROCHETE (BORRELIA BURGDORFERI). *J Parasitol* 86, 1223–1228 (2000).

73. Bouzid, N. M., Archie, J. W., Anderson, R. A., Grummer, J. A. & Leaché, A. D. Evidence for ephemeral ring species formation during the diversification history of western fence lizards (Sceloporus occidentalis). *Mol Ecol* 31, 620–631 (2022).

74. Barbour, A. G. Isolation and cultivation of Lyme disease spirochetes. *Yale J Biology Medicine* 57, 521–5 (1984).

75. Takacs, C. N. *et al.* A CRISPR Interference Platform for Selective Downregulation of Gene Expression in Borrelia burgdorferi. *Appl Environ Microb* 87, e02519-20 (2021).

76. Gust, K. A. *et al.* Multiple environmental stressors induce complex transcriptomic responses indicative of phenotypic outcomes in Western fence lizard. *Bmc Genomics* 19, 877 (2018).

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