UNIVERSITY OF CALIFORNIA
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Unraveling the Tick-Host-Pathogen Interface

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Entomology

by

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ABSTRACT OF THE DISSERTATION

Unraveling the Tick-Host-Pathogen Interface

by

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Doctor of Philosophy, Graduate Program in Entomology
University of California, Riverside, August 2013
Dr. Joao Pedra, Chairperson

Vector-borne diseases have a major impact in mortality and morbidity throughout the tropics. Vector-borne pathogens are transmitted to humans by blood feeding arthropods like mosquitoes and ticks. A true understanding of the vector-borne disease cycle requires the study of the major players involved in this cycle: the mammalian host, the pathogen and the arthropod vector. This thesis examines these aspects by using a tick-borne disease – human granulocytic anaplasmosis (HGA) – as a model. Chapters 1, 2 and 3 emphasize the importance of vector-borne diseases and discuss medically relevant arthropod vectors in light of their immune response to human pathogens. The etiologic agent of HGA, *Anaplasma phagocytophilum*, is also examined in detail, with a focus on immune evasion strategies used to colonize mammals and ticks. Chapters 4 thru 6 are dedicated to address pathogen, host and the tick vector, respectively. First, the contribution of one *A. phagocytophilum* gene – the dihydrolipoamide dehydrogenase – to infection is demonstrated. Next, the role of tick saliva as an immunomodulator of *A. phagocytophilum* infection in the mammalian host is revealed. Finally, an *Ixodes scapularis* X-linked inhibitor of apoptosis protein is described as an E3 ubiquitin ligase that controls tick colonization by *A. phagocytophilum*. In summary, this dissertation uncovers three distinct events underlying the tick-pathogen-host interface.

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Chapter 1: A Brief Introduction to Vector-borne Diseases

Vector-borne diseases such as malaria, dengue and West Nile encephalitis are caused by the transfer of pathogenic microorganisms from a vector (e.g., a mosquito, a tick) to a mammalian host. They represent a significant part of the neglected diseases that affect the health of billions of people worldwide. In fact, vector-borne pathogens are estimated to infect a significant percentage of the world's population (Table 1) (Hotez, Fenwick et al. 2009). Mosquitoes are the most important arthropod vector of human diseases, followed by ticks, sandflies, tsetse flies and kissing bugs. Blackflies and lice are also critical in terms of transmission of vector-borne diseases to humans.

Epidemiologists, physicians, vector biologists, microbiologists and immunologists have long studied the many aspects implicated in the vector-borne disease cycle. One may assume that a disruption in the vector-borne disease cycle ultimately leads to the prevention of disease transmission to humans. Therefore, researchers have engaged in the search for new and alternative ways to promote that. This thesis analyses different aspects involved in the vector-pathogen-host triad with the aim to further contribute to the study of the vector-borne diseases that have devastated the lives of billions of people worldwide. Here, host, pathogen and arthropod vector will be carefully addressed, with a special emphasis in the tick-borne disease used as model – human granulocytic anaplasmosis.

Table 1. Vector-borne diseases of importance based upon health and economical impact.

Disease	Pathogen	Vector	Estimated number of Affected Individuals (Per Year)	Mortality
1. Malaria	*Plasmodium spp.*	*Anopheles gambiae*	216 million	655, 000
2. Lymphatic filariasis	*Wuchereria bancrofti* *Brugia spp.*	*Culex spp.* *Anopheles spp.* *Aedes spp.* *Mansonia spp.*	120 million	---
3. Dengue fever	Dengue virus	*Aedes aegypti* *Aedes albopictus*	50 million	22,000
4. Leishmaniasis	*Leishmania spp.*	*Lutzomyia longipalpis*	12-15 million	60, 000
5. Chagas disease	*Trypanosoma cruzi*	*Rhodnius prolixus*	10 million	> 10,000
6. Yellow fever	Yellow fever virus	*Aedes aegypti*	200,000	30, 000
7. Lyme Borreliosis	*Borrelia burgdorferi*	*Ixodes spp.*	110,000 **	1 **
8. West Nile neuroinvasive disease	West Nile virus	*Culex quinquefasciatus*	*	*

* Estimates are not available

** Estimates in the United States and Europe

--- Not applicable

Chapter 2: Vector immunity, the Barrier to Bypass

2.1 Abstract

Vector-borne diseases are illnesses that affect the health of billions of people worldwide and are caused by the transfer of a pathogenic microorganism from a vector to a mammalian host. For vector-borne pathogens, the innate immune system defines the type and magnitude of host and vector response. It also plays an important role towards arthropod-borne pathogen recognition. Pioneering studies in *Drosophila* and mosquito immunity have elucidated immunological signaling pathways in arthropods. This chapter will review the major signaling pathways involved in the humoral response of arthropod vectors in the light of the most medically relevant human pathogens they transmit.

2.2 Introduction to vector immunology

In order for vector-borne diseases to occur, an arthropod vector must first successfully acquire and later transmit a pathogenic microorganism to a mammalian host. In other words, if an arthropod is not capable of hosting a microorganism within its system and provide the appropriate conditions for pathogen colonization, the vector-borne disease cycle will not perpetuate. This is a complex process and one of the key aspects influencing arthropod suitability is its immune response to an invading pathogen. This chapter will cover vector humoral immunity, with a special emphasis on the current research describing how human pathogens colonize arthropod vectors.

2.3 The *Drosophila* model

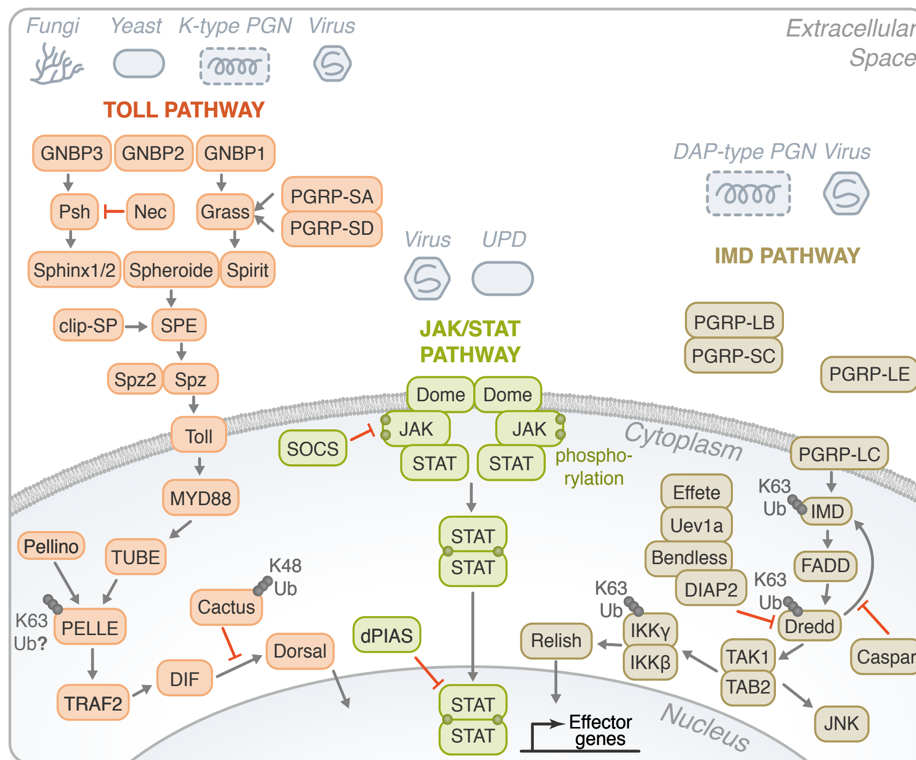
Much of what is known about immune responses in invertebrates, and even in vertebrates, comes from the use of insect models over the past 20 years. For a number of reasons, fruit flies are the most well studied insect system, and the most widely used model organism for research purposes. In order to analyze the information available for other arthropods, the *Drosophila* immune system must be briefly addressed first (please refer to Figure 1).

The Toll pathway is an evolutionarily conserved cascade involved in the establishment of the dorsal-ventral axis in *Drosophila melanogaster* (Anderson, Bokla et al. 1985). It is also activated in the presence of Gram-positive bacteria and fungi (Lemaitre, Nicolas et al. 1996; Lemaitre, Reichhart et al. 1997), as well as in the

presence of viruses (Zambon, Nandakumar et al. 2005). Unlike the human Toll-like receptors, Toll does not bind directly to pathogens or pathogen-related particles but to a cytokine, Spätzle. The downstream core cascade is, nevertheless, similar to that of mammals. In the hemolymph, pro-Spätzle is directly cleaved to its active form by a processing enzyme. Upon bacterial challenge with Gram-positive bacteria, the detection of lysine-containing peptidoglycan (PGN) is mediated by Peptidoglycan recognition proteins (PGRPs), PGRP-SA and PGRP-SD, and by a Gram-negative binding protein. Once Spätzle is activated, it binds to Toll and induces its dimerization (Weber, Tauszig-Delamasure et al. 2003). This step leads to the recruitment of three Death domain-containing molecules: the myeloid differentiation primary response gene (MyD88) and Tube, which seem to already exist as a complex, and Pelle, a kinase homologous to the mammalian Interleukin (IL)-1 receptor-associated kinase (IRAK) family of kinases. Activation of Pelle leads to the degradation of Cactus, the *Drosophila* inhibitor of κB homolog (Lemaitre, Nicholas et al. 1996). This causes the release of the Rel transcription factors Dorsal-related immunity factor (Dif) and Dorsal, members of nuclear factor kappa B (NF-κB) family (Ip, Reach et al. 1993). These molecules cross the membrane and reach the nucleus, where they induce transcription of different immune responsive genes, such as drosomycin and defensins.

The Imd pathway is another important pathway in humoral immunity. It was named after a mutation called *imd* (immune deficiency) (Lemaitre, Kromermetzger et al. 1995), which led to the disruption of expression of antibacterial peptides. The *imd* gene was found to encode a Death domain-containing protein that shares homology with the receptor interacting protein (RIP) of the mammalian tumor necrosis factor receptor pathway. This cascade is activated by mono-diaminopimelic acid (DAP)-type

peptidoglycans, present mostly in Gram-negative bacteria, but also in Gram-positives like *Bacillus* spp. These DAP-type PGNs are sensed by the PGRP-LC and PGRP-LE receptors (Kaneko, Yano et al. 2006). Ligand-induced clustering, dimerization or multimerization of these receptors appear to be critical steps in the signaling cascade. Once this occurs, the IMD adaptor is recruited and interacts with Fas-Associated protein with Death Domain (FADD), which binds the Death related ced-3/Nedd2-like (Dredd) caspase (Hu and Yang 2000). As a consequence, the ubiquitin E3-ligase *Drosophila* inhibitor of apoptosis protein 2 (DIAP2) is also recruited and targets Dredd for K63-linked polyubiquitination (Meinander, Runchel et al. 2012). Active Dredd cleaves IMD, which leads to the exposure of its neo N-terminal allows the association of the cleaved IMD and DIAP2 (Paquette, Broemer et al. 2010). This association provides the conjugation of IMD and K63 polyubiquitin chains in a process involving the E2 ligases Effete, Bendless and Uev1a (Zhou, Silverman et al. 2005; Paquette, Broemer et al. 2010). This step seems to be crucial for the activation of TGF-β activated kinase (TAK1) and the *Drosophila* homolog of the mammalian I-kappa B kinase (IKK) complex (Silverman, Zhou et al. 2000; Vidal, Khush et al. 2001). Next, TAK1 forms a complex with the adaptor molecule TAB2 that binds to the K63 polyubiquitin chains, leading to the induction of the two branches of the IMD pathway: c-Jun N-terminal kinases (JNK) and NF-κB. The JNK branch of the IMD pathway is involved mainly in stress response and wound healing. The NF-κB branch is directly related to the expression of AMPs. TAK1 activates *Drosophila* IKK complex, which phosphorylates Relish, a bipartite molecule similar to the mammalian NF-κB precursors. The cleavage of Relish by a caspase releases its N-terminal Rel homologous domain transcription factor that translocates into the nucleus and lead to the transcription of immune genes (Stoven, Silverman et al.


Fungi
Yeast
K-type PGN
Virus
Extracellular Space
TOLL PATHWAY
GNBP3
GNBP2
GNBP1
Psh
Nec
Grass
PGRP-SA
PGRP-SD
Sphinx1/2
Spheroide
Spirit
clip-SP
SPE
Spz2
Spz
Toll
MYD88
Pellino
TUBE
K63 Ub?
PELLE
TRAF2
DIF
Cactus
K48 Ub
Dorsal
Virus
UPD
JAK/STAT PATHWAY
Dome
Dome
JAK
JAK
SOCS
STAT
STAT
phospho-rylation
STAT
STAT
dPIAS
STAT
STAT
DAP-type PGN Virus
IMD PATHWAY
PGRP-LB
PGRP-SC
PGRP-LE
Cytoplasm
PGRP-LC
K63 Ub
IMD
Effete
Uev1a
Bendless
DIAP2
FADD
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IKKγ
IKKβ
Relish
TAK1
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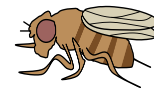
JNK
Effector genes
Nucleus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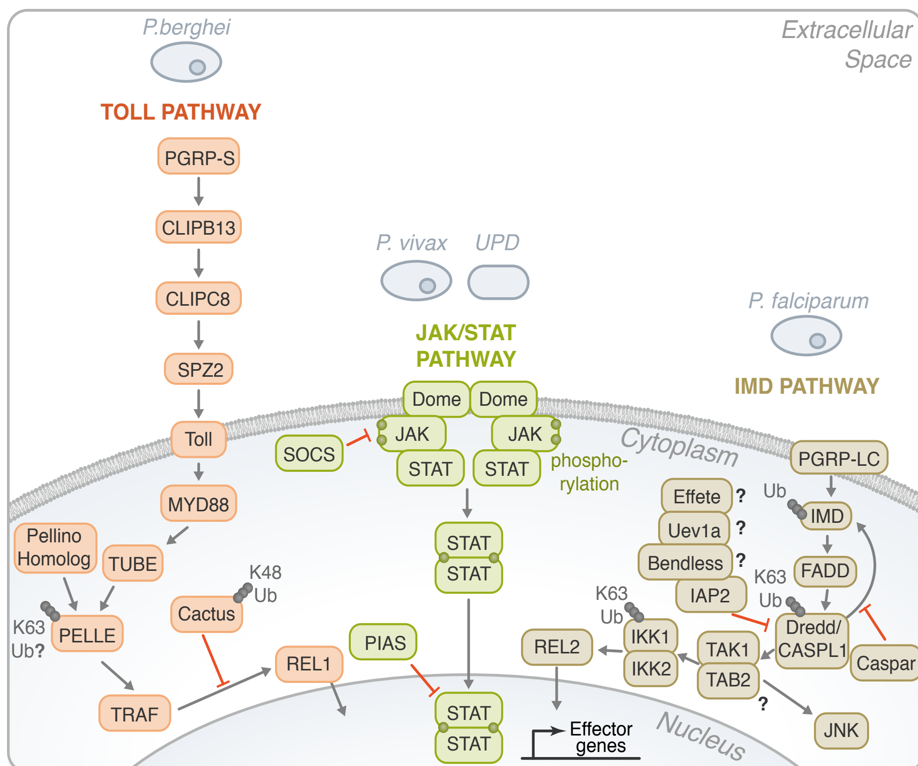
P.berghei
Extracellular Space
TOLL PATHWAY
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Toll
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PELLE
TRAF
Cactus
K48 Ub
REL1
P. vivax
UPD
JAK/STAT PATHWAY
Dome
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JAK
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SOCS
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phospho-rylation
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PIAS
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P. falciparum
IMD PATHWAY
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PGRP-LC
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IAP2
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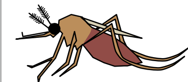
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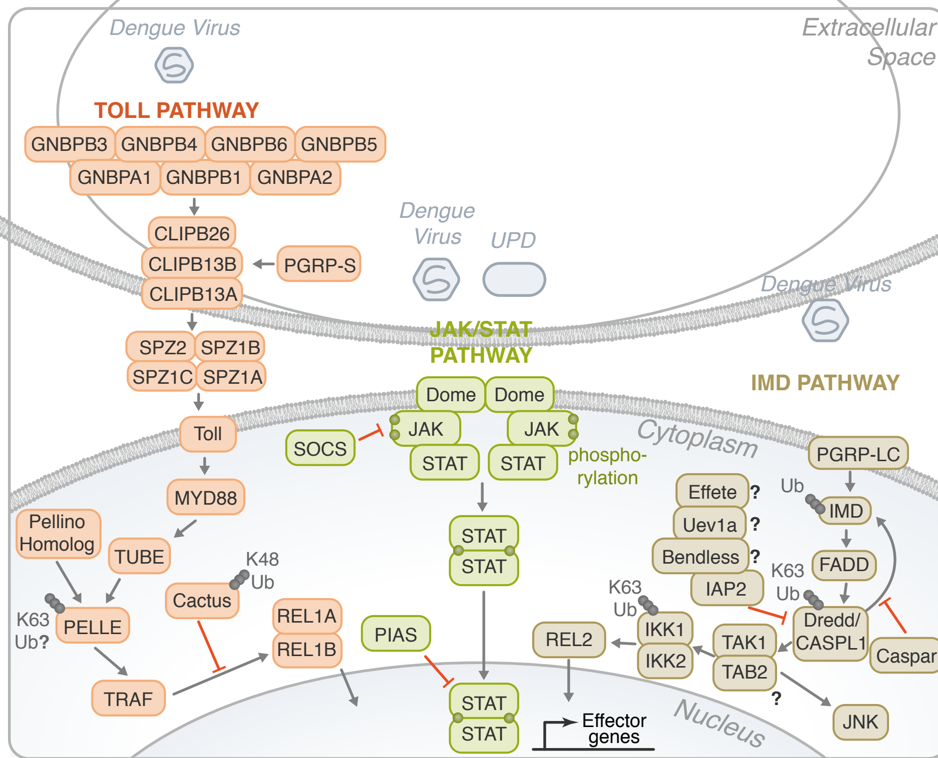
Dengue Virus
Extracellular Space
TOLL PATHWAY
GNBPB3
GNBPB4
GNBPB6
GNBPB5
GNBPA1
GNBPB1
GNBPA2
CLIPB26
CLIPB13B
PGRP-S
CLIPB13A
Dengue Virus
UPD
Dengue Virus
SPZ2
SPZ1B
SPZ1C
SPZ1A
JAK/STAT PATHWAY
IMD PATHWAY
Dome
Dome
Toll
SOCS
JAK
JAK
Cytoplasm
PGRP-LC
STAT
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phospho-rylation
MYD88
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K48 Ub
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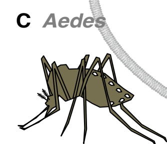
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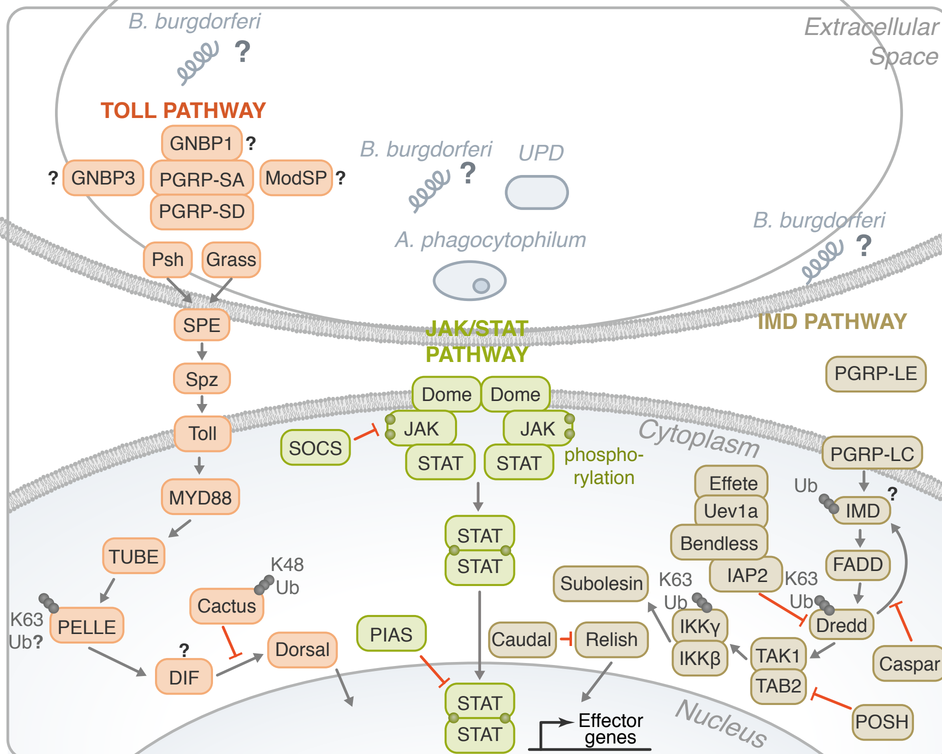
B. burgdorferi ?
Extracellular Space
TOLL PATHWAY
GNBP1 ?
? GNBP3
PGRP-SA
ModSP ?
B. burgdorferi ?
UPD
PGRP-SD
B. burgdorferi ?
A. phagocytophilum
Psh
Grass
SPE
JAK/STAT PATHWAY
IMD PATHWAY
Spz
PGRP-LE
Dome
Dome
Toll
SOCS
JAK
JAK
Cytoplasm
PGRP-LC
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phospho-rylation
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Nucleus

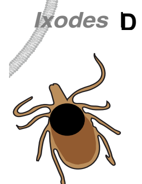
Ixodes D

Figure1: Conservation of the Toll, IMD and JAK/STAT pathways in *Drosophila melanogaster, Anopheles gambiae, Aedes aegypti* and *Ixodes scapularis.* (A) In *Drosophila,* Gram-negative bacteria-binding proteins (GNBPs) and peptidoglycan recognition proteins (PGRPs) have been shown to activate the Toll pathway in the presence of stimulants, such as fungi, yeast, lysine-type peptidoglycan (K-type PGN) and viruses. These recognition proteins signal downstream to Persephone (psh) and Grass. CLIP domain serine proteases (clip-SP) modulate the signaling after recognition as well. Sphinx1/2, Spheroide and Spirit initiate the activation of Spätzle through the Spätzle processing enzyme (SPE). Spätzle binds to Toll which recruits three Death domain-containing molecules: MyD88, Tube and Pelle. Pellino/Pellino homologue, perhaps, acts as a positive regulator of immunity by ubiquitinating Pelle. After which, TNF-receptor-associated factor (TRAF) signals to Dorsal-immune related factor (DIF), followed by signaling to Dorsal. The activation is facilitated by the degradation of Cactus through K48 ubiquitination. The transcription factor translocates to the nucleus in order to up-regulate immune genes. The Toll pathway is highly conserved in: (B) *Anopheles,* (C) *Aedes* and (D) *Ixodes.* The Toll pathway has been demonstrated to recognize *Plasmodium berghei* in (B) *Anopheles* and the Dengue virus in (C) *Aedes.* TRAF signaling initiates REL1 and REL1A/1B activity in (B) *Anopheles* and (C) *Aedes* respectively. (A) On the other hand, the *Drosophila* IMD pathway recognizes primarily mono-diaminopimelic acid-type peptidoglycans (DAP-type PGN). Fas-associated protein with death domain (FADD) is recruited to IMD. FADD binds to the Death related ced-3/Nedd2-like caspase (Dredd)/CASPL1. Dredd can cleave IMD. Inhibitor of apoptosis (IAP) can also associate with Dredd/CASPL1. Effete, Uev1a and Bendless play a role in the regulation of this step and Caspar may also inhibit the activity of IMD-dependent transcription factors. TGF-b activated kinase (TAK1), TAK1-binding protein 2 (TAB2) complex forms as signaling continues. Two avenues may result from the IMD pathway: JNK or NF-κB. For NF-κB activation, Relish translocates to the nucleus to activate effector genes. There are several potential sites of ubiquitination throughout the IMD pathway: IMD, Dredd/CASPL1 and the IKK complex. Like the Toll pathway, the IMD pathway is found in many species: (B) *Anopheles,* (C)

Aedes and (D) *Ixodes. P. falciparum* and the dengue virus can trigger the IMD pathway in (B) *Anopheles* and (C) *Aedes* respectively. While Relish is regulated by the IMD pathway in (A) *Drosophila* and (D) *Ixodes,* REL2, the homologue of Relish, acts as the transcription factor in (B) *Anopheles* and (C) *Aedes.* The third pathway is the JAK/STAT pathway. (A) A ligand derived from the unpaired (UPD) gene activates the pathway by binding to Domeless (Dome). Phosphorylated JAK promotes the dimerization of STAT. Dimerized STAT can proceed to the nucleus. Countering the activation, both SOCS and PIAS negatively regulate the JAK/STAT pathway. Although the JAK/STAT pathway is evolutionarily conserved across the organisms discussed, various pathogens have demonstrated the ability to activate the JAK-STAT pathway, such as: (B) *Plasmodium vivax,* (C) dengue virus and (D) *A. phagocytophilum.* For the Toll, IMD and JAK/STAT pathways, *B. burgdorferi* recognition in *I. scapularis* remains mostly elusive.

2003). Recent evidences indicate that the Imd-mediated expression of an antimicrobial peptide also controls antiviral responses (Huang, Kingsolver at al. 2013).

A third pathway, named the Janus kinase and signal transducers and activators of transcription (JAK/STAT) pathway, is also present in fruit flies. It involves the unpaired ligand, transmembrane receptor Domeless (Dome), the JAK Hopscotch and the STAT transcription factor. This pathway is implicated in cytokine production in mammals and was first shown to be important for insect immunity in *Anopheles*. The binding of unpaired to Dome leads to a conformational change in this receptor and a subsequent self-phosphorylation of JAK/Hop (Harrison, McCoon et al. 1998). Once this enzyme is activated, it phosphorylates Dome and the docking sites for STATs are formed. STATs

are then recruited and also phosphorylated, and their dimerization leads to translocation into the nucleus, where they will lead to the transcription of target genes. A protein inhibitor of activated STAT (PIAS) and a suppressor of cytokine signaling in Drosophila regulate this pathway. The genes encoding the complement-like protein *Tep2* and the *Turandot* stress genes in *Drosophila* are controlled by this pathway (Ekengren and Hultmark 2001), but their transcription also seems to involve the mitogen-activated protein kinase (MAPK) pathway. Studies have shown that flies produce antiviral molecules in a JAK/STAT-dependent manner, as for the *Drosophila* C virus-responsive genes (Dostert, Jouanguy et al. 2005).

RNA interference (RNAi) is one of the signaling pathways that endogenously control gene expression. In invertebrates, it is also known to control antiviral responses (Sabin, Zheng et al. 2013; Chotkowski, Ciota et al. 2008). RNA silencing starts with the RNase III-like enzyme Dicer (Dcr), which releases a 21-23nt RNA molecule from the previous larger dsRNA that triggered the antiviral response. This small interfering RNA (siRNA) is incorporated into an effector complex, the RNA-induced silencing complex (RISC), and is retained to guide RISC in cleaving the complementary sequence on the mRNA target, a viral RNA species. The siRNA that is produced in response to a viral RNA is called a virus-derived siRNA (vsiRNA) (Aliyari, Wu et al. 2008). Mutations in the core components of the siRNA machinery implicates in sensitivity to a range of RNA viruses (van Mierlo, Bronkhorst et al. 2012; Zambon, Vakharia et al. 2006; Nayak, Berry et al. 2010; Mueller, Gausson et al. 2010). Recently, the response to a DNA virus was also shown to involve RNA silencing (Bronkhorst, van Cleefet al. 2012; Kemp, Mueller et al. 2013).

The biogenesis of vsiRNAs is mediated by Dcr-2 and plays an essential role in the antiviral response through RNAi silencing. This enzyme belongs to the DExD/H-box helicase family, as do the mammalian RIG-I-like receptors that sense and respond to cytoplasmic viral RNA (Deddouche, Matt et al. 2008). The antiviral molecule Vago was described to be crucial against viral replication in *Drosophila*, and its expression is dependent on Dcr-2. The exact mechanism by which Dcr-2 controls the expression of Vago is still not clear (Deddouche, Matt et al. 2008). The processing of the siRNA also depends on a protein named r2d2, which forms a complex with Dcr-2, and a recently identified protein, Ars2, required for the efficient dsRNA processing (Sabin, Zhou et al, 2009). Interestingly, the Argonaute-2 endonuclease, a co-effector of and essential for antiviral responses in *Drosophila* (van Rij, Saleh et al. 2006), is inhibited by Cricket Paralysis Virus (Nayak, Berry et al. 2010). This illustrates that viruses have evolved virulence effectors to evade the host RNAi defense. For some vectors of viral diseases, more information is also discussed below.

2.4 Mosquitoes: the major vectors

Mosquitoes are the most important vectors of human diseases in the world. *An. gambiae* immunity has been the focus of several studies to further understand the mechanisms underlying *Anopheles*-*Plasmodium* interactions. There are more than 150 named species of *Plasmodium* that infect various species of vertebrates. Four species are considered parasites of humans: *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. Another species, *P. berghei*, a rodent malaria agent, has been used for research purposes as a model. As for *Drosophila* and vertebrates, mosquitoes recognize

pathogens by pathogen recognition receptors (PRRs), which identify pathogen-associated molecular patterns, or PAMPs. Several immune-related gene families have been suggested to encode PRRs, such as leucine-rich repeats proteins (LRRs) and thioester-containing proteins (TEPs). TEP1, which is a complement-like protein comparable to the vertebrate C3, as well as LRR immune protein 1 (LRIM1) and the amyloid-precursor-like 1 C (APL1C) have been described as anti-parasitic factors (Blandin, Shiao et al. 2004) (Povelones, Waterhouse et al. 2009). Two NF-κB transcription factors, Rel1 and Rel2, orthologous to *Drosophila* Dorsal and Relish, respectively, have been described in the *Anopheles* genome. No ortholog for *Drosophila* Dif was found in this mosquito genome. Knockdown of Rel2 and its regulator, Caspar showed that Rel2 controls *An. gambiae* resistance to *P. falciparum* (Garver, Dong et al. 2009). This Rel1/Rel2-dependent response regulates the basal expression of the major anti-parasitic factors, such as TEP1. The JAK/STAT pathway also seems to be involved in limiting *P. berghei* and *P. falciparum* multiplication in the mosquito midgut (Gupta, Molina-Cruz et al. 2009). *S*erine protease inhibitors (serpins), which regulate Toll activation, for example, as well as CLIP domain proteases, implicated in antimicrobial synthesis in *Drosophila* (Ligoxygakis, Pelte et al. 2002) have been already identified as regulators of proteolytic cascades in *Anopheles*.

Besides *Anopheles*, *Aedes aegypti* is another important mosquito species of medical relevance. It transmits yellow fever and dengue fever, both caused by flaviviruses, as well as the parasitic disease filariasis. Dengue is the most common human arthropod-borne viral ailment. In *Ae. aegypti,* two isoforms of Rel1, Rel1-A and -B, seem to cooperate in enhancing gene expression (Waterhouse, Kriventseva et al. 2007). A long and predominant isoform of Rel2, similar to *Drosophila* Relish, and a short

Rel2 that lacks ankyrin and Death domains have been described. Rel1 seems to be directly related to the Toll pathway and its activation in response to DENV infection since an up-regulation of Rel1 and of its upstream putative PRRs, together with its downstream AMPs, was demonstrated (Xi, Ramirez et al. 2008). Moreover, it has been observed that Toll signaling controls anti-dengue response independently of *Aedes* strain or DENV serotypes, reinforcing the importance of this pathway against dengue infection (Xi, Ramirez et al. 2008).

The RNAi pathway also modulates the DENV-2 infection of *Ae. aegypti*. This pathogen appears to have co-adapted to *Ae. aegypti* RNAi response in order to persist in the vector, which leads to a longer-term survival of the infected mosquito (Sanchez-Vargas, Scott et al. 2009). Furthermore, the importance of JAK/STAT pathway to control dengue infection was shown by using RNAi to deplete a negative regulator, PIAS. This pathway represents an independent cascade of anti-dengue response since none of the immune genes that are co-regulated by DENV-2 and Toll pathways were observed to be regulated by PIAS depletion (Souza-Neto, Sim et al. 2009).

West Nile virus (WNV) is a RNA virus of re-emerging importance. *Culex* spp. mosquitoes transmit it, and WNV infection may cause life-threatening meningoencephalitis or long-term neurologic sequelae in humans. Nevertheless, little is known about the West Nile-mosquito interactions. *Culex quinquefasciatus* gene family members have been shown to share large similarities with *A. aegypti* (Bartholomay, Waterhouse et al. 2010), suggesting that common antiviral mechanisms exist in WNV-*Culex* system. C-type lectins are PRRs known for their key importance in recognizing pathogen-derived carbohydrates. An *Ae. aegypti* C-type lectin, mosGCLT-1, was found

not only to be induced upon WNV infection but also to interact with WNV and facilitate its entry. *Culex mosGCTL-1* was also up-regulated by WNV infection *in vivo* and its RNAi silencing reduced the WNV burden as well (Cheng, Cox et al. 2010). These findings have allowed the formulation of a model in which secreted *mosGCTL*-1 found in the hemolymph would bind to WMN and function as an extracellular receptor that facilitate and enhance viral spread throughout the mosquito body. In mosquito cells, *Culex* ortholog of Vago is up-regulated in response to WNV infection in a Dcr-2-dependent manner. Secretion of this peptide also activates the JAK/STAT pathway restricts WNV infection (Paradkar, Trinidad et al, 2012). Artificial infection of *Culex pipiens quinquefasciatus* induces the RNAi pathway. Interestingly, the WNV genome regions more strongly targeted by RNAi were found more likely to hold point mutations when compared to weakly targeted areas (Brackney, Beane et al. 2009). Additional studies are necessary to uncover more processes involved the WNV infection of mosquitoes *in vitro* and *in vivo*.

2.5 Kissing bugs, sandflies, tsetse flies and fleas: the minority

For many years, kissing bugs, especially *Rhodnius prolixus,* were widely used as a model to understand more about insect endocrinology. Recently, insect immunity has also been addressed. Cellular and humoral responses, whose arrangement is very similar to *Drosophila*'s, have been recently reported using *Trypanosoma rangeli* as a model organism. This protozoan is harmless in humans, and different results were obtained for *Trypanosoma cruzi,* the etiological agent of Chagas disease. Against this latter pathogen, encapsulation and nodulation play the major role since no genes related

to humoral responses were found to be up-regulated in the response to it (Ursic-Bedoya and Lowenberger 2007). A study has also suggested that *T. cruzi* modulates *R. prolixus* microbiota to increase host colonization (Castro, Moraes et al. 2012).

In sandflies, most of what has been analyzed relates to the modulation of host immune response by saliva. Nonetheless, few genes implicated in the innate immunity of the key vector of *Leishmania* in the Americas, the sand fly *Lutzomyia longipalpis*, have been identified by EST sequencing (Ramalho-Ortigao, Temporal et al. 2001). A defensin, a glycin-rich protein, as well as a PGRP and a serpin were found in the genome of this phlebotomine. Although they are believed to be up-regulated after a blood meal, their precise relationship with *Leishmania* is still unclear (Pitaluga, Beteille et al. 2009). Using RNAi, a recent paper has also identified a Caspar-like gene that regulates *Leishmania* infection of *L. longipalpis* (Telleria, Sant'Anna et al. 2012). The same group showed that a *L. longipalpis* defensin gene is modulated by bacterial feeding and injection, as well as by *Leishmania* infection (Telleria, Sant'Anna et al. 2013).

Fleas are also blood-feeding vectors whose physiology is not well understood. *Yersinia pestis*, the causative agent of plague, regulates gene expression both in the flea and in mammals. However, how fleas respond to the presence of this pathogen is very poorly known. A recent work reported the up-regulation of several genes in the flea, some of which were predicted to have a role in innate immunity based on similarity to mammalian immune defense. Moreover and interestingly, *Y. pestis* obtained from infected fleas showed more resistance to phagocytosis by macrophages *in vitro*, when compared to bacteria grown *in vitro* (Vadyvaloo, Jarrett et al. 2010). *Y. pestis* was

described to require a plasmid-encoded phospholipase D named as *Yersinia* murine toxin for its survival in the midgut of the rat flea *Xenopsylla cheopis,* its principal vector (Hinnebusch, Rudolph et al. 2002).

Tsetse flies (*Glossina* spp.) are the sole vector of African trypanosomes, *Trypanosoma brucei*, the agent of African sleeping sickness in sub-Saharan Africa. Studies have shown that these flies posses both the Toll and IMD pathways as major pillars of humoral responses, and trypanosome infection activates the IMD pathway (Lehane, Aksoy et al. 2003). However, different *Glossina spp.* shows distinct patterns of competence in transmitting this protozoan. Attacin, cecropin and defensin have been observed in the hemolymph of *Glossina morsitans morsitans* tsetse flies upon ingestion of *T. b. brucei* (Harrington 2011). In fact, it has been indicated that the level of attacin, specifically, plays a central role in determining susceptibility to trypanosome. Refractory tsetse flies constitutively expression attacin systemically, whereas susceptible flies fail to show such pattern before blood meal ingestion (Nayduch and Aksoy 2007). Refractoriness to trypanosome infection also correlates with PGRP-LB levels. Moreover, trypanosome infection increases in the midgut of *G. morsitans* upon knockdown of attacin, Relish, PGRP-LB, or PGRP-LC (Wang, Wu et al. 2009; Harrington 2011).

2.6 Ticks: the other side

The term vector-borne diseases also include diseases transmitted by non-insect arthropods like ticks. In fact, ticks can be considered second only to mosquitoes as vectors of disease-causing agents to humans, transmitting a diversity of pathogens. For

example, they transmit *Anaplasma phagocytophilum*, which causes human granulocytic anaplasmosis, and *Borrelia burgdorferi*, the etiologic agent of Lyme disease, which is the most frequently reported vector-borne disease in the United States. In fact, ticks surpass mosquitoes and other blood-feeding vectors in terms of the variety of pathogens they can transmit. This arthropod has a well-developed innate immune system, but only very little has been recognized about tick immunity, especially when compared to other vectors. In terms of humoral responses, the following AMPs are known in ticks: lectins, defensins, lysozymes, proteases and protease inhibitors. Defensins are the best characterized of these and many defensins have been identified in different species of ticks (Hynes, Ceraul et al. 2005; Todd, Sonenshine et al. 2007). In general, tick defensins are very similar to insect defensins and comparison of the protein sequences of defensins from tick and other invertebrates showed a high conservation.

Ixodes scapularis, also known as the deer tick, is the primary vector of *A. phagocytophilum* and *B. burgdorferi* in the United States (Pagel Van Zee, Geraci et al. 2007), and probably the major tick species used for medical research purposes. Comparative genomics approaches have illustrated that the core set of genes involved with the Toll, IMD, JAK/STAT, and RNAi pathways is conserved in *I. scapularis* relative to *Drosophila* and mosquitoes (unpublished data). Mechanistic studies, nevertheless, are still limited and much needed to uncover the regulation of AMP expression upon pathogen colonization of tick tissues, among many other aspects of tick-pathogen interactions.

In *Dermacentor variabilis,* a hard tick capable of transmitting several human bacteria, such as *Francisella tularensis*, the etiologic agent of tularemia, defensins were

found to be up-regulated in response to *B. burgdorferi.* Injection of this bacterium, however, did not affect defensin expression in its major tick vector, *I. scapularis* (Hynes, Ceraul et al. 2005). Interestingly, a *B. burgdorferi* strain lacking outer surface protein A and B cannot colonize the tick gut. In order to infect *I. scapularis*, *B. burgdorferi* utilizes a receptor in the tick gut called TROSPA, which recognizes an outer surface protein A heavily expressed by these spirochetes (Pal, Li et al. 2004). This was the first report of arthropod ligands required by these spirochetes. Furthermore, an *I. scapularis* tick histamine release factor (tHRF) was observed as critical for tick engorgement and *B. burgdorferi* transmission to the murine host since RNAi silencing impaired tick feeding and, consequently, pathogen inoculation. Similar results were also achieved with immunization studies using a recombinant tHRF or passive immunization (Dai, Narasimhan et al. 2010). Silencing of other *B. burgdorferi*-induced genes, such as *salp15*, also reduces tick *B. burgdorferi* transmission to mice (Ramamoorthi, Narasimhan et al. 2005). Another tick protein, salp16, seems to be required for tick infection with *A. phagocytophilum*. The interactions between ticks and *A. phagocytophilum* - the tick-borne pathogen used as a model in this dissertation work - will be discussed in detail in the following chapter.

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Chapter 3: *A. phagocytophilum* as a Model

3.1 Abstract

A. phagocytophilum is an obligate intracellular rickettsial pathogen transmitted by ixodid ticks. This bacterium colonizes myeloid and nonmyeloid cells and causes human granulocytic anaplasmosis – an important immunopathological vector-borne disease in the USA, Europe and Asia. Recent studies uncovered novel insights into the mechanisms of *A. phagocytophilum* pathogenesis and immunity. Here, an overview of the underlying events by which the immune system responds to *A. phagocytophilum* infection, how this pathogen counteracts host immunity and the contribution of the tick vector for microbial transmission are discussed in details.

3.2 Introduction to *A. phagocytophilum*

The word *Anaplasma* comes from the Greek *an*, which means “without”, and *plasma*, “anything formed or molded”. This genus comprises microorganisms that show no specific form, and were once thought to be viruses because of their small size and intracellular life cycle. In fact, they are true bacteria of both veterinary and medical interest and structurally similar to Gram-negative microorganisms. *A. phagocytophilum* is probably the most relevant of them in terms of human health - the etiologic agent of human granulocytic anaplasmosis (Rikihisa 2010). First identified as *Ehrlichia equi* and *E. phagocytophila*, this rickettsial pathogen was recently placed in the newly formed family Anaplasmatacaea, order Rickettsiales (Figure 2). This family includes both pathogenic and nonpathogenic obligate intracellular bacteria that are confined within membrane bound compartments in the host cytoplasm and are maintained in animal and arthropod reservoirs.

In the United States, *I. scapularis* is the most important tick species transmitting *A. phagocytophilum* to humans. Once *I. scapularis* bites an infected wild reservoir, such as white-footed mice or raccoons, it acquires *A. phagocytophilum* and will become capable of transmitting it to other animals, including humans, after metamorphosis into nymphal or adult ticks. In fact, humans are mere accidental hosts of *A. phagocytophilum*. Nevertheless, infection by this pathogen leads to the development of a disease called human granulocytic anaplasmosis (HGA). The clinical signs of HGA include fever, headache and myalgia, with leukocytosis and increased level of hepatic aminotransferases. The severity of the symptoms varies from asymptomatic to mortality,

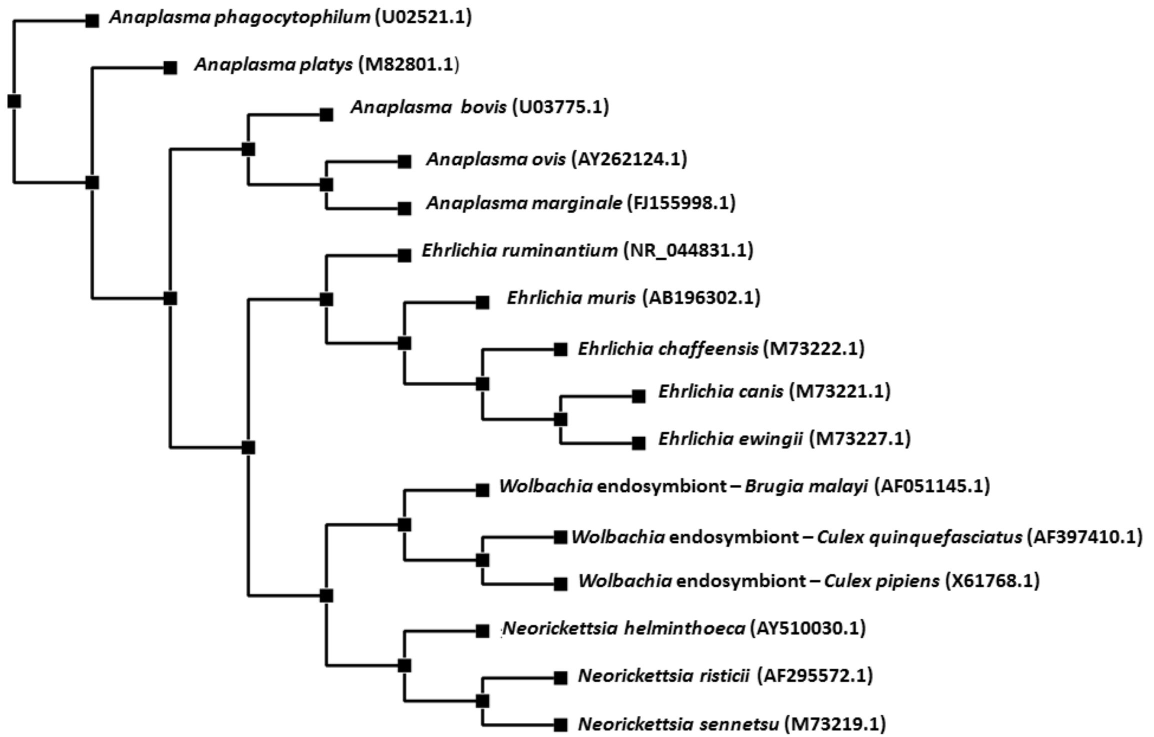

Figure 2: Anaplasmataceae phylogenetic tree. The order Rickettsiales, family Anaplasmataceae includes bacteria, such as: *Anaplasma spp., Ehrlichia spp., Wolbachia spp.* and *Neorickettsia spp*. The Anaplasmataceae phylogenetic tree was built according to a maximum likelihood based on SEQBOOT alignment of 16S ribosomal RNA gene sequences utilizing POWER (http://power.nhri.org.tw/power/home.htm). Accession numbers were obtained from GenBank.

and the disease is usually considered self-limiting (Thomas, Dumler et al. 2009). Treatment relies on the use of the broad-spectrum antibiotic doxycycline, but this illness

can evolve to severe and potentially fatal conditions. The underlying causes of these fatal episodes, however, are unknown and misdiagnosis remains a common occurrence despite the effort of many professionals. Thankfully, recent advances in molecular techniques have allowed researchers to properly identify and understand more about how this unusual pathogen successfully invade and proliferate inside neutrophils, causing a systemic disease. Studies have also helped uncovering how *A. phagocytophilum* colonizes ticks in nature. Such remarkable ability is only possible due to the evolution of mechanisms that allow pathogen survival within the hostile environment of their hosts. How *A. phagocytophilum* evades host and vector immune responses to promote its survival as well as the colonization of both its arthropod vector and its mammalian host will be covered below.

3.3 *A. phagocytophilum* genomics and host regulation

The *A. phagocytophilum* HZ strain has a genome size of 1.47 Mb comprising around 12% of repetitive sequences and with about 1,300 open reading frames, most of which encode for housekeeping genes (Dunning Hotopp, Lin et al. 2006). Although this bacterium does not show ATP/ADP translocase or cytochrome d-type oxidase genes, it does contain a partial glycolysis pathway. It is also capable of synthesizing all nucleotides and most vitamins and co-factors, but only four amino acids (Dunning Hotopp, Lin et al. 2006). Interestingly, it lacks genes necessary for synthesis of lipopolysaccharide (LPS) or PGN, and because of that the *A. phagocytophilum* membrane stability is reduced. *A. phagocytophilum* does not produce cholesterol.

Instead, cholesterol from the mammalian host is “hijacked” to promote membrane stability, growth and survival (Lin and Rikihisa 2003).

Cholesterol is acquired by *A. phagocytophilum* from the low density lipoprotein-mediated uptake pathway and not by “de novo” synthesis (Xiong, Lin et al. 2009). Proteins known as sterol regulatory element binding proteins (SREBP) are transcription factors involved in regulating cholesterol-mediated feedback to maintain appropriate cholesterol homeostasis. SREBPs do not respond to the increase in cholesterol during *A. phagocytophilum* infection. Rather, there is a post-transcriptional mechanism that regulates LDL receptor expression in human HL-60 cells. This causes cholesterol to accumulate, which in turn facilitates *A. phagocytophilum* replication inside cells. The endosomal transmembrane protein the Niemann-Pick Type C1 (NPC1) can be observed in *A. phagocytophilum* inclusions. NPC1 is involved in the uptake of cholesterol and membrane synthesis, and RNA interference experiments have demonstrated that it is necessary for pathogen infection (Xiong and Rikihisa ; Lin and Rikihisa 2003). Finally, *A. phagocytophilum* infection of leukocytes is also inhibited by treatment with methyl-β-cyclodextrin, which extracts cholesterol (Lin and Rikihisa 2003).

A. phagocytophilum expresses a variety of 44 kDa immunodominant proteins encoded by the *p44/msp2* multigene family (Lin, Zhang et al. 2006). The p44/msp2 proteins are major outer membrane proteins with porin activity and the *A. phagocytophilum* genome possesses 113 *p44/msp2* loci with truncated or short 5’ or 3’ fragments, several of which appear to function as donor sequences for conversion at the dominant expression locus. *A. phagocytophilum* lacks the RecBCD recombination pathway and uses the RecF pathway at a single expression locus for homologous

recombination (Lin, Zhang et al. 2006). This recombination makes *A. phagocytophilum* capable of expressing a range of specific proteins on its surface to avoid host immune response, contributing to its persistence within the intracellular environment.

A. phagocytophilum uses a Type IV secretion system (T4SS), which is an ATP-dependent system to secrete proteins or DNA from the bacteria to the eukaryotic cell. Expression of T4SS in *A. phagocytophilum* is tightly regulated to allow secretion of specific substrates that affect the host cell metabolism. *A. phagocytophilum* T4SS is composed of *virB* genes and this pathogen has up to eight distinct copies (Rikihisa and Lin 2010). *A. phagocytophilum* infected ISE6 and HL-60 cells have been shown to have differential transcription of *virB2* homologues (Nelson, Herron et al. 2008). Two T4SS effector molecules have been identified: ankyrin-repeat-rich A (AnkA) and *Anaplasma* translocated substrate 1 (Ats-1). *A. phagocytophilum* infection stimulates phosphorylation of the AnkA tyrosine, which is mediated by the interaction of AnkA with Abl-1 via Abl interactor 1 (Abi-1). Phosphorylated AnkA then interacts with the host tyrosine phosphatase, Src homology phosphatase-1 (SHP-1) (JW, Carlson et al. 2007). Treatment with an Abl kinase-specific inhibitor STI571 as well as Abl-1 siRNA abrogates *A. phagocytophilum* infection (Lin, den Dulk-Ras et al. 2007).

Ats-1 is highly expressed by *A. phagocytophilum* during infection of human cells. It translocates five membranes to reach the mitochondria and inhibit apoptosis (Niu, Kozjak-Pavlovic et al. 2010) by inhibiting etoposide-induced cytochrome c release and poly ADP-ribose polymerase cleavage. Recently, Ats-1 was demonstrated to bind to BECN1 and induce the formation of autophagosomes. Fusion of autophagosomes and *A. phagocytophilum* inclusions provides this bacterium with nutrients necessary for its

growth (Niu and Rikihisa 2013). Another protein, APH_0032, was recently observed to accumulate in the *A. phagocytophilum* vacuole but it seems not to be actively secreted by T4SS (Huang, Troese et al. 2010).

3.4 *A. phagocytophilum* binding and colonization

During the tick bite, *A. phagocytophilum* gains access to the bloodstream and soon reaches the intracellular environment necessary for its replication and host colonization. Besides infecting circulating leukocytes, the presence of *A. phagocytophilum* has also been linked to endothelial cells (Munderloh, Lynch et al. 2004), and it has been speculated that infecting the endothelium may serve as an initial step after *A. phagocytophilum* transmission and before granulocyte infection. *In vitro* studies using human microvascular epithelial cells (HMEC) demonstrated that *A. phagocytophilum* could invade and grow within HMEC-1 cells and transfer from these to neutrophils when co-incubation is allowed. This model has been suggested because *A. phagocytophilum* up-regulates the ICAM-1 leukocyte adhesion molecule in infected HMEC-1 cells (Herron, Ericson et al. 2005), and binds to ligands used by granulocytes to roll on inflamed endothelium, such as P-selectin glycoprotein ligand-1 (PSGL-1) (Choi, Garyu et al. 2003). Additionally, this pathogen induces release of IL-8 from human neutrophils. This chemokine recruits neutrophils to the site of infection, which can be targets of microbial invasion and further propagation (Akkoyunlu, Malawista et al. 2001). *A. phagocytophilum* binding also decreases neutrophil migration and diapedesis on inflamed endothelium (Schaff, Trott et al. 2010), which may, in turn, inhibit inflammation

signaling and facilitate the establishment of *A. phagocytophilum* inside a mammalian host.

Different research groups have illustrated the complexity of *A. phagocytophilum* binding and infection in different mammalian systems. An example is the use of tetrasaccharide sialyl Lewis (sLe^x) present on PSGL-1, which is required for human infection (Goodman, Nelson et al. 1999; Herron, Nelson et al. 2000). Conversely, *A. phagocytophilum* use α-1,3-fucosylation but not PSGL-1 for infection of murine neutrophils (Carlyon, Akkoyunlu et al. 2003). Similarly, sialylated glucans are not required for endothelial cells infection (Herron, Ericson et al. 2005), and different *A. phagocytophilum* isolates may use PSGL-1-dependent and independent routes to infect myeloid cells (Sarkar, Reneer et al. 2007). In HL-60 cells, *A. phagocytophilum* binding results in activation of the PSGL-1 signaling pathway, leading to phosphorylation of ROCK1 by spleen tyrosine kinase (Syk) (Figure 3). ROCK1 is a serine/threonine kinase that regulates actin organization. Therefore, it has been speculated that actin reorganization through ROCK1 activation could facilitate *A. phagocytophilum* invasion of these cells. Moreover, *A. phagocytophilum* entry requires signaling platforms, such as: lipid rafts and caveolin-1. These molecular structures co-localize with early inclusions of *A. phagocytophilum* in HL-60 cells (Lin and Rikihisa 2003). Its role in entry and infection, however, is elusive. Clathrin is dispensable for *A. phagocytophilum* internalization, whereas glycosylphosphatidylinositol (GPI) - anchored proteins (GAP) and flotillin 1 have been found to be necessary for *A. phagocytophilum* binding to mammalian host cells (Figure 3) (Lin and Rikihisa 2003). The signaling cascades triggered downstream of these events remain poorly understood.

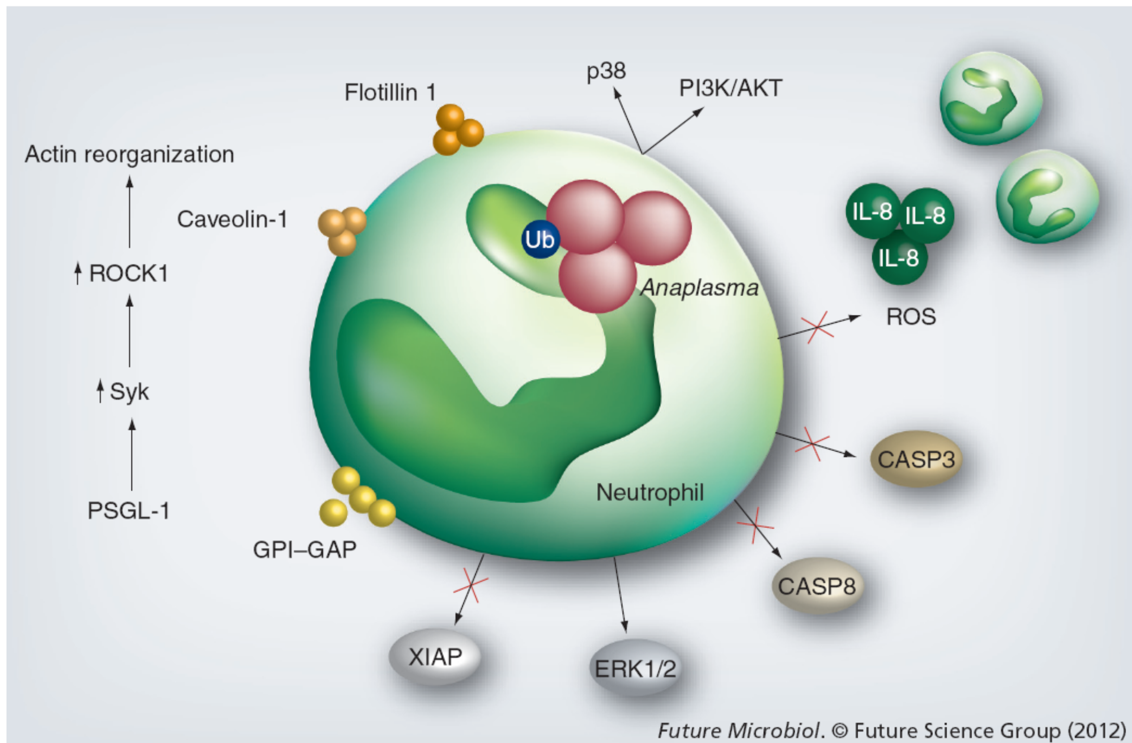

Figure 3: *A. phagocytophilum* modulates the host machinery. *A. phagocytophilum* infection of human cells causes IL-8 secretion, which leads to the recruitment of neutrophils. Neutrophil apoptosis is inhibited through degradation of the x-linked inhibitor of apoptosis protein (XIAP) and dampening of apoptotic caspase function, such as: caspases 3 and 8 (CASP3/8). The p38 MAP kinase and the pI3K/AKT signaling pathways are involved in this process. Reactive oxygen species (ROS) production is inhibited by modulating NADPH oxidase assembly and/or regulation of gene expression. The ERK pathway is also affected by this pathogen. PSGL1 signaling is activated during infection leading to actin reorganization via the molecules Syk and ROCK1. *A. phagocytophilum* entry also requires lipid rafts, caveolin-1, glycoinositol phospholipid anchored proteins (GPI-GAP) and flotillin1. Recently, mono-ubiquitination (Ub) was shown to decorate the *A. phagocytophilum* vacuole.

The vacuole where *A. phagocytophilum* is found is not entirely isolated from the host cell; instead, it recruits molecules associated with membrane trafficking in order to camouflage and attain the nutrition required for survival. In fact, *A. phagocytophilum* itself has 41 genes with functions associated with protein binding and transport (Dunning Hotopp, Lin et al. 2006). Moreover, human proteins associated with cytoskeleton, trafficking, signaling and energy metabolism were shown to be up-regulated in HL-60 cells infected with *A. phagocytophilum* when compared to non-infected cells (Lin and Rikihisa 2003), indicating that this microorganism interferes with host vesicular trafficking to persist in vacuoles. These membrane-bound inclusions do not seem to be endosome-like since they lack major endosomal markers, such as early endosomal antigen 1, transferrin receptor and annexins I, II, IV and VI. In addition, they are not acidic and do not accumulate myeloperoxidases, CD63, lysosomal-associated membrane protein (LAMP)-1 or Golgi vesicular makers (Lin and Rikihisa 2003). *A. phagocytophilum* morulae have several hallmarks of autophagosomes including a double lipid bilayer and co-localization with LC3 and Beclin-1, ATG8 and ATG6 (Niu, Yamaguchi et al. 2008). *A. phagocytophilum* employs Rab GTPases associated with recycling endosomes that appears to facilitate pathogen survival (Huang, Hubber et al. 2010).

Many pathogens that reside inside vacuoles are known to exploit the ubiquitination system since this post-translational modification is an important step in modulating many cellular processes. Ubiquitination is a cell specific process where a 76 amino acid protein is covalently attached to a lysine of a target protein. Three main enzymes are involved in this process, an ubiquitin activating enzyme (E1), the E2 ubiquitin conjugating enzyme and an E3 ubiquitin protein ligase (Price and Kwaik 2010; Fujita and Yoshimori 2011). Mono- and poly-ubiquitination are the two main types of

ubiquitination that can occur and intracellular bacterial pathogens have been shown to secrete effectors that mimic E3 ubiquitin ligase activity to regulate protein levels (Price and Kwaik 2010; Fujita and Yoshimori 2011). Interestingly, a recent study showed that membranes of the *A. phagocytophilum* vacuole accumulate mono-ubiquitin during mammalian infection of myeloid and endothelial cells. Mono-ubiquitin accumulates to a lesser extent in ISE6 cells (Huang, Ojogun et al. 2011). Nevertheless, how *A. phagocytophilum* affects host ubiquitination and colonization remains unknown.

3.5 *A. phagocytophilum* and humans: just an accident

How does HGA happen?

Human outdoor activities, especially during the warmer spring and summer months, may lead to tick exposure and *A. phagocytophilum* infection. If tick feeding and pathogen transmission takes place successfully, symptoms such as fever, chills, headache and muscle aches will appear within 1 or 2 weeks after a tick bite (Dumler 2012). The underlying events that lead to disease manifestation are not well understood. A remarkable feature of HGA is that it does not result from direct pathogen load. Evidence corroborating with this assessment includes leucopenia seen as a major hallmark of *A. phagocytophilum* infection in humans, in which more leukocytes are lost than could be accounted for by a direct role of *A. phagocytophilum* infection. Consistently, recent findings have pointed out to a correlation between clinical signs and host-derived immunopathology (Dumler 2012).

Polymorphonuclear leukocytes (PMN) play a major role in oxygen defense systems, and are mainly used to destroy phagocytosed bacteria by producing reactive oxygen species (ROS). Production of ROS, however, can also lead to tissue injury and this is the case for *A. phagocytophilum*. Superoxide generated by NADPH oxidase during *A. phagocytophilum* infection causes damaging inflammatory histopathology (Lepidi, Bunnell et al. 2000; Martin, Caspersen et al. 2001; Scorpio, Von Loewenich et al. 2005; Browning, Garyu et al. 2006; Scorpio, von Loewenich et al. 2006; Choi, Webb et al. 2007). Decreased bone marrow function and changes in hematopoietic progenitor and peripheral blood cells in the spleen have been described in acute infection with *A. phagocytophilum* (Johns, Macnamara et al. 2009). This has been associated with an aberrant CXCL12/CXCR4 signaling as well as hematopoietic stem cell mobilization (Johns and Borjesson) and bone marrow production of myelosuppressive chemokines (Borjesson, Macnamara et al. 2009).

Studies characterizing cytokine response to *A. phagocytophilum* infection indicate that the response weights towards the T helper (Th) 1 phenotype. Interferon (IFN)-γ and IL-10 levels are elevated, whereas tumor necrosis factor (TNF)-α, IL-4 and IL-6 seem to be unchanged during the acute phase of HGA in the blood (Dumler, Trigiani et al. 2000). This is consistent with a lack of increased p38 mitogen-activated protein kinase (MAPK) in neutrophils exposed to *A. phagocytophilum* (Kim and Rikihisa 2002). Infection with *A. phagocytophilum* leads to increased levels of IL-8, as well as of other chemokines, but not of IL-6 or TNF-α by HL-60 and bone marrow cells (Klein, Hu et al. 2000). High levels of IL-8 recruit neutrophils, and granulocytic phagocytosis is normally increased by IL-8, which can facilitate *A. phagocytophilum* dissemination.

Blocking of the IL-8 receptor CXCR1 causes a decrease in the pathogen load but that does affect the host pathology (Scorpio, Caspersen et al. 2004).

In the early phase of infection, IL-12/23p40 regulates $CD4^+$ T cells, while IL-12/23p40-independent mechanisms contribute to pathogen elimination from the host (Pedra, Tao et al. 2007). IL-18 produced by the inflammasome, a protein scaffold associated with the inflammatory process, also regulates $CD4^+$ T cell responses (Pedra, Sutterwala et al. 2007). Expression of toll-like receptors (TLRs) or Myd88 remains unaltered during *A. phagocytophilum* infection of neutrophils (Pedra, Sukumaran et al. 2005), and the c-Jun NH2-terminal kinase 2 (JNK2) inhibits production of IFN-γ by NK cells upon *A. phagocytophilum* challenge in mice (Pedra, Mattner et al. 2008). Splenic CD8 T lymphocytes, which are activated during infection, have also been associated with IFN-γ levels and hepatic inflammatory lesions (Dumler 2012) as well as phosphorylation of STAT1 (Choi and Dumler 2013). In IFN-γ knockout mice, bacterial levels in the tissues are increased in the early phase of infection, but tissue damage is absent and bacteria are eventually eliminated. The same study described increased lesions in IL-10 knockout mice, which showed normal levels of IFN-γ (Akkoyunlu and Fikrig 2000).

To date, mice deficient in TLR2, TLR4, iNOS, MyD88, TNF and NADPH oxidase have been studied and they are all capable of clearing *A. phagocytophilum* infection (von Loewenich, Baumgarten et al. 2003). CD11b/CD18, on the other hand, seems to be crucial for bacterial clearance because infection of CD11b/CD18 knockout mice leads to an increase in bacterial load when compared to wild-type mice (Borjesson, Simon et al. 2002). Furthermore, the NLRC4, but not the NLRP3 inflammasome, is partially required

for *A. phagocytophilum* host response *in vivo* (Pedra, Sutterwala et al. 2007) and illustrates how complex the immune response to *A. phagocytophilum* can be. There is also a dichotomy in *A. phagocytophilum* infection: neutrophils are not efficient in clearing infection, and innate immune responses are the major mediators of HGA pathogenesis, whereas adaptive immunity represented by $CD4^+$ T-cell activation and IFN-γ production controls pathogen eradication. How these two branches come together as a whole in the response to *A. phagocytophilum* remains a matter of much debate.

Down-regulation of oxidative and inflammatory responses

Microarray analysis in neutrophils (Sukumaran, Carlyon et al. 2005), together with a more current proteomic analysis in HL-60 cells (Lin, Kikuchi et al. 2011), indicated that genes and proteins involved in innate immunity and inflammation have their expression modulated by *A. phagocytophilum* infection. Neutrophils are the most abundant type of phagocyte and the major mediator of the respiratory burst activated upon exposure to pathogens. *A. phagocytophilum* does not code for genes involved in detoxification and does not induce ROS when infecting murine or human neutrophils (Rikihisa 2010). Moreover, this pathogen inhibits mRNA expression of $gp91^{phox}$ [also known as NOX2], and decreases $P22^{phox}$ protein levels, while leaving other components of this system unaffected in human neutrophils (Banerjee, Anguita et al. 2000; Carlyon, Chan et al. 2002). When *A. phagocytophilum* is released, infected cells are reported to scavenge for exogenous superoxide, which cannot diffuse through an intact bacterial inner membrane (Carlyon, Abdel-Latif et al. 2004).

Once infected with *A. phagocytophilum*, neutrophils become refractory to stimuli such as LPS and phorbol myristate acetate (PMA) (Banerjee, Anguita et al. 2000; Wang, Malawista et al. 2002), but this active inhibition is not seen in human monocytes (Mott and Rikihisa 2000). In fact, *A. phagocytophilum* is easily killed when exposed to ROS and this might explain why this pathogen does not infect circulating monocytes. Using an *A. phagocytophilum* mutant lacking the dihydrolipoamide dehydrogenase 1 (*lpda1*) gene, Chen and colleagues found neutrophils to be refractory to *lpda1*::TnHimar1 mutant infection, with a much lower NF-κB response compared to the wild-type strain. Macrophages, on the other hand, had higher levels of NF-κB signaling and were fully capable of binding and internalizing the *lpda1*::TnHimar1 mutant (Chen, Severo et al. 2012). *A. phagocytophilum* promotes distinct signals between neutrophils and monocytes (Kim and Rikihisa 2002; Chen, Severo et al. 2012) in terms of cytokine generation, reinforcing the role of specific subsets of cells in the *A. phagocytophilum* colonization of mammalian hosts.

In HL-60 cells, *A. phagocytophilum* prevents the assembly of NADPH oxidase subunits (Mott, Rikihisa et al. 2002; Carlyon, Abdel-Latif et al. 2004), and also down-regulates NOX2 and surface protein levels (Banerjee, Anguita et al. 2000). Downregulation of NOX2 have been associated with the production of AnkA by *A. phagocytophilum*, which has been shown to bind to the CYBB/NOX2 locus (Park, Kim et al. 2004; Garcia-Garcia, Rennoll-Bankert et al. 2009). Activation of nuclear cathepsin L and enhanced binding of CCAAT displacement protein (CDP) have also been described during *A. phagocytophilum* infection of neutrophils (Thomas, Samanta et al. 2008). Furthermore, *A. phagocytophilum* minimizes the release of proinflammatory cytokines in

human peripheral blood and HL-60 cells (Klein, Hu et al. 2000). Inhibition of TNF-α, IL-6 and IL-13 has been reported in *A. phagocytophilum*-infected mast cells (Ojogun, Barnstein et al. 2011), suggesting that mitigation of mast cell activation can also contribute to *A. phagocytophilum* subversion of host defenses. Chromatin modifications within the host cell have been linked to host gene transcription during *A. phagocytophilum* infection, and gene expression can also be regulated through histone acetylation. Histone modifying enzymes, such as histone deacetylases (HDAC), maintain histone modification patterns. The up-regulation of HDAC together with the epigenetic silencing of host cell defense genes has been described as required for *A. phagocytophilum* infection of THP-1 cells (Garcia-Garcia, Barat et al. 2009).

Subversion of host apoptosis and autophagy

Neutrophils generally have a very short half-life, which makes it surprising that *A. phagocytophilum* would find them suitable to inhabit. To survive in a hostile environment such as inside neutrophils, intracellular pathogens like *A. phagocytophilum* are prompt to evade apoptosis. *A. phagocytophilum* inhibits neutrophil apoptosis long enough to develop the morula (Rikihisa 2010). *A. phagocytophilum* infection up-regulates expression of anti-apoptotic *blc-2* genes, blocks cell surface Fas clustering during spontaneous neutrophil apoptosis, and inhibits cleavage of pro-caspase 8 and caspase 8 activation (Pedra, Sukumaran et al. 2005; Lee and Goodman 2006). Inhibition of Bax translocation into the mitochondria, in addition to activation of caspase 9 and degradation of X- linked inhibitor of apoptosis protein (XIAP), a caspase inhibitor, have

also been reported (Ge and Rikihisa 2006). As previously mentioned, Ats-1 is secreted by the *A. phagocytophilum* T4SS and prevents mitochondria from responding to apoptotic signals.

Autophagy works in synchrony with the host immune response due to its role in clearing intracellular infections. *A. phagocytophilum* inclusions display a range of autophagosome markers and do not mature into autolysosomes. Indeed, *A. phagocytophilum* infection is favored by treatment with rapamycin, an autophagy inducer, but treatment with 3-methyladenine, which inhibits autophagy, reversibly arrests *A. phagocytophilum* growth without preventing pathogen internalization. This indicates that *A. phagocytophilum* infection is aided by subverting autophagy (Niu, Yamaguchi et al. 2008). Taken together, *A. phagocytophilum* manipulates host cell machinery to induce autophagy and cytoplasmic recycling for its own development.

Activation of protein kinases

A. phagocytophilum infection also activates protein kinase pathways in the host. The AnkA tyrosine is progressively phosphorylated during *A. phagocytophilum* intracellular growth cycle and this phosphorylation has been associated with the activation of two host tyrosine kinases, Arc and Abelson leukemia 1(Ab1) (Rikihisa 2010). Ab1 activity was shown as crucial for *A. phagocytophilum* infection, and the extracellular signal regulated kinase (ERK) pathway is activated by *A. phagocytophilum* in neutrophils. *A. phagocytophilum* toxin A (AptA) activates Erk1/2 phosphorylation and co-localizes with the intermediate filament protein, vimentin. This protein is reorganized

around *A. phagocytophilum* inclusions and is necessary for ERK1/2 activation, but further studies are needed to address the precise mechanism behind this observation (Sukumaran, Mastronunzio et al. 2011).

3.6 The tick interface: so close but yet so far

Tick-borne pathogens have evolved to share an intimate relationship with their hosts. Nonetheless, the precise co-evolutionary history of *Ixodes* spp. and *A. phagocytophilum* remains unclear. Once acquired via blood meal, *A. phagocytophilum* reaches the gut and later migrates to the salivary glands allowing transmission and continuity of its life cycle. This is only possible due to an orchestrated pattern of gene expression regulating pathogen development and host physiology. In order to survive and perpetuate this cycle, *A. phagocytophilum* has to control expression of its own genes, but it must also alter gene expression in ticks. Researchers have only started to understand these events using tick cell lines and *in vivo* approaches. The most recent results describing *I. scapularis* - *A. phagocytophilum* interactions are summarized below.

The P11 protein was recently shown to be required for *A. phagocytophilum* migration and hemocyte infection (Liu, Narasimhan et al. 2011) (Figure 4). Another molecule affected by *A. phagocytophilum* infection is the salivary gland protein SALP16. *A. phagocytophilum* up-regulates *salp16* to survive within the tick vector (Sukumaran, Narasimhan et al. 2006) and alters the monomeric/filamentous (G/F) actin ratio leading to translocation of phosphorylated/G-actin to the nucleus (Sultana, Neelakanta et al. 2010). This selectively regulates *salp16* gene transcription in association with the RNA

polymerase II (RNAPII) and the TATA-binding protein. Strikingly, *A. phagocytophilum* failed to induce actin phosphorylation in primary cultures of human neutrophils, suggesting that this phenomenon is specific for the arthropod vector. *A. phagocytophilum* seems to also inhibit apoptosis and promote cytoskeleton rearrangement to invade tick cells.

When in nature, ticks often have to survive extreme conditions, such as low humidity and temperatures. Fikrig and colleagues recently demonstrated that *A. phagocytophilum* appears to increase *I. scapularis* ability to survive in cold temperatures by up-regulating an antifreeze glycoprotein (Neelakanta, Sultana et al. 2010). This would, in turn, allow *A. phagocytophilum* to survive in cold temperatures. α1, 3-fucosyltransferases are also up-regulated in ticks during *A. phagocytophilum* infection. When α1, 3- fucosyltransferases are silenced *in vivo*, *A. phagocytophilum* is less efficient at colonizing ticks because a decrease in pathogen acquisition during feeding was observed. Pathogen transmission, however, was unaffected indicating that *A. phagocytophilum* uses α1, 3-fucose specifically for acquisition (Pedra, Narasimhan et al. 2010). On the other hand, the tick salivary protein subolesin was down-regulated during *A. phagocytophilum* infection of *I. scapularis* nymphs but the same was not observed in ISE6 cells. Additionally, vaccination against subolesin was protective against tick infection (de la Fuente, Blouin et al. 2008). The mechanism by which subolesin contributes to *A. phagocytophilum* pathogenesis is still unresolved. Heat shock proteins (HSPs) are also involved in tick response to feeding and *A. phagocytophilum* infection. Busby *et al.* 2011 (Busby, Ayllon et al. 2011) demonstrated the involvement of HSP20

and subolesin in the tick response to *A. phagocytophilum*. They hypothesized that another HSP, HSP70, may be manipulated by *A. phagocytophilum* to increase infectivity.

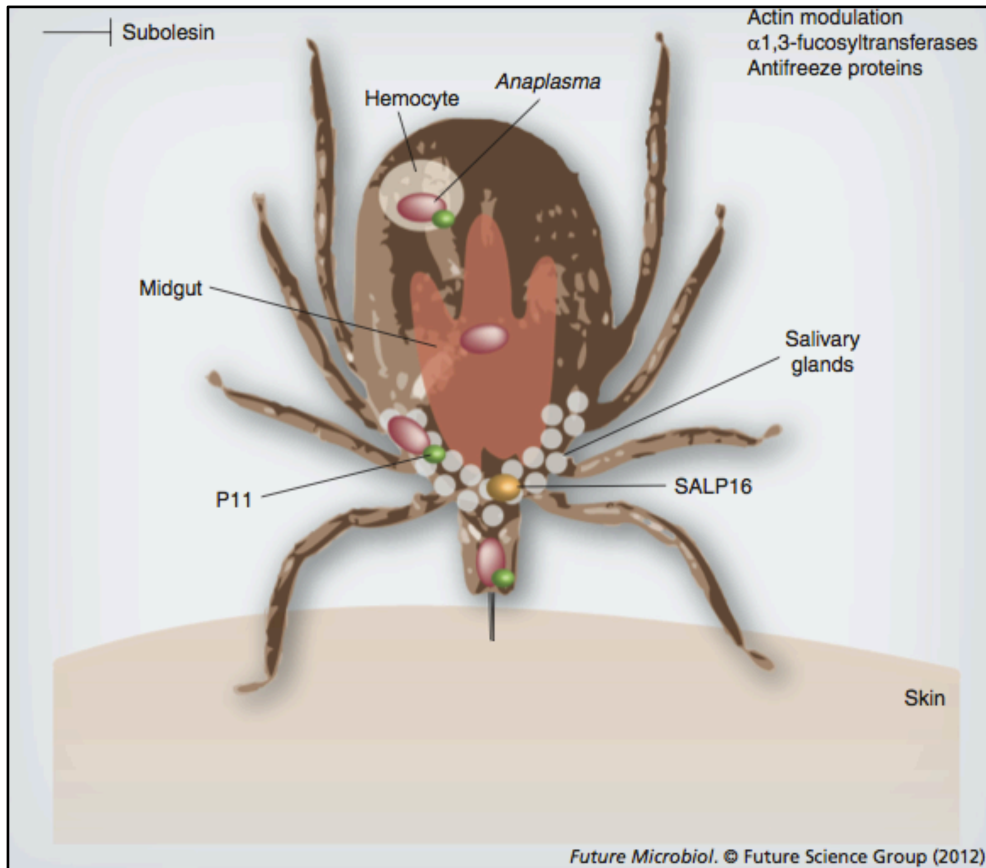

Figure 4. *A. phagocytophilum* manipulates the tick vector for its own benefit. The tick *I. scapularis* pierces the skin using its hypostome. During feeding, *A. phagocytophilum* alters *I. scapularis* gene expression for colonization, enters the midgut and migrates to the salivary glands via hemocytes. Bioactive molecules, such as P11, bind to *A. phagocytophilum* during hemocyte colonization and facilitate pathogen trafficking to the salivary glands. *A. phagocytophilum* inhibits tick subolesin and modulates the expression of a tick salivary protein named salp16 for its own survival. *A. phagocytophilum* also induces actin phosphorylation leading to the translocation of

phosphorylated G-actin to the nucleus. Up-regulation of antifreeze proteins favors tick survival in cold temperatures. When α1,3-fucosyltransferases are silenced by siRNA, *I. scapularis* acquisition of *A. phagocytophilum* is decreased, suggesting that α1,3-fucosylated structures are critical for pathogen colonization.

Transcription profiling of *A. phagocytophilum* show a possible role for *virB2* genes during tick infection. These genes code for a surface-exposed pilus and are part of the *A. phagocytophilum* T4SS. Using tilling arrays, Munderloh and colleagues have found that *virB2* genes show human- or tick cell-dependent differential transcription. Moreover, *A. phagocytophilum* has human- and tick-specific operons and paralogs, such as for the major surface proteins *p44/msp2* (Nelson, Herron et al. 2008). Another study analyzed the *A. phagocytophilum* expression profile during infection of ISE6 cells and found that this pathogen clearly modulates tick gene expression (Zivkovic, Blouin et al. 2009). *A. phagocytophilum* morulae can be individually detected in HL-60, but not in ISE6 cells, in which *A. phagocytophilum* appears enlarged and pleomorphic (Munderloh, Jauron et al. 1999). These findings underscore the existence of specific adaptations to divergent hosts and suggest that this bacterium uses different strategies to colonize tick and mammalian cells. Distinct events underlying the *A. phagocytophilum* cycle – its ability to immunomodulate mammalian immunity and promote infection, as well as its acquisition by the tick host – will be further addressed in the incoming chapters.

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Chapter 4: *A. phagocytophilum* Dihydrolipoamide Dehydrogenase Gene

4.1 Abstract

A. phagocytophilum is a tick-borne rickettsial pathogen that provokes an acute inflammatory response during mammalian infection. The illness caused by *A. phagocytophilum*, human granulocytic anaplasmosis, occurs irrespective of pathogen load and results instead from host-derived immunopathology. Thus, characterizing *A. phagocytophilum* genes that affect the inflammatory process is critical for understanding disease etiology. Here, a single transposon insertion into the *A. phagocytophilum* dihydrolipoamide dehydrogenase 1 gene (*lpda1 [APH_0065*]) is shown to affect inflammation during infection. *A. phagocytophilum* lacking *lpda1* revealed altered clinicopathological abnormalities during mammalian colonization. Hence, LPDA1 is described as an important immunopathological molecule during *A. phagocytophilum* infection.

4.2 Introduction

Bacterial pathogens use a range of effectors to exploit host cells in order to promote survival and evade host immune responses. These microorganisms may subvert cell death and apoptosis for their dissemination and propagation inside host cells. They may also inhibit or prevent oxidative responses and even "hijack" host cell nutrients necessary for their metabolism. An example of bacterial pathogen capable of performing such tasks is *A. phagocytophilum*. These characteristics, together with other peculiarities related to *A. phagocytophilum* immune evasion and host colonization have already been discussed in Chapter 3.

Many of the aspects underlying *A. phagocytophilum* immune evasion and host colonization have been described in previous studies for different microorganisms and systems. Interestingly, emerging literature indicates that proteins involved in central and intermediary metabolism are also implicated in bacterial virulence in a number of important human pathogens, including *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa* (Henderson and Martin 2011; Venugopal, Bryk et al. 2011; Hallstrom, Morgelin et al. 2012). Here, an *A. phagocytophilum* bacterial enzyme involved in intermediary metabolism is reported for the first time as a potential bacterial virulence effector and host immunomodulatory factor. *A. phagocytophilum* dihydrolipoamide dehydrogenase 1 (LPDA1) affected inflammation during infection in mice. These findings suggest that LPDA1, a critical component of the α-ketoacid dehydrogenase intermediary metabolism identified throughout Anaplasmataceae and Rickettsiae families of bacteria, acts as an immunopathological molecule during *A. phagocytophilum* infection.

4.3 Results

A. phagocytophilum lpda1 expression during pathogen infection

Phenogram analysis revealed that *lpda* genes are present across many members of the Anaplasmataceae and Rickettsiaceae families, including *A. phagocytophilum* (Figure 5A). The *A. phagocytophilum* HZ genome was found to carry three *lpda* genes, two of which are 100% similar (*APH_0065* and *APH_1070*) and it is unclear whether they are paralogs (Figure 5B). A third gene (*APH_0393*) is clustered together with the *lpda* sequence from the closely related species *A. marginale* (*AM_797*) (Figure 5B). Further investigation of *lpda1* (*APH_0065*) showed that this gene carries three domains: two Rossmann-fold $NAD(P)^+$-binding protein domains (NADB Rossmann); positions 2 – 40 (e-value = 3.73 e^{-5}); 185 – 243 (e-value: 3.89 e^{-9}) and one pyridine nucleotide-disulphide oxidoreductase dimerization domain (pyridine oxidoreductase); positions 352 – 461 (e-value = 4.26 e^{-33}) (Figure 5C). *Lpda1* expression was low in *I. scapularis* ticks, mouse neutrophils and human PBMCs during bacterial colonization (Figure 5D). However, *lpda1* was highly expressed in the human promyelocytic leukemia cell line HL-60, dendritic cells (BMDDC) and macrophages (BMDMs).

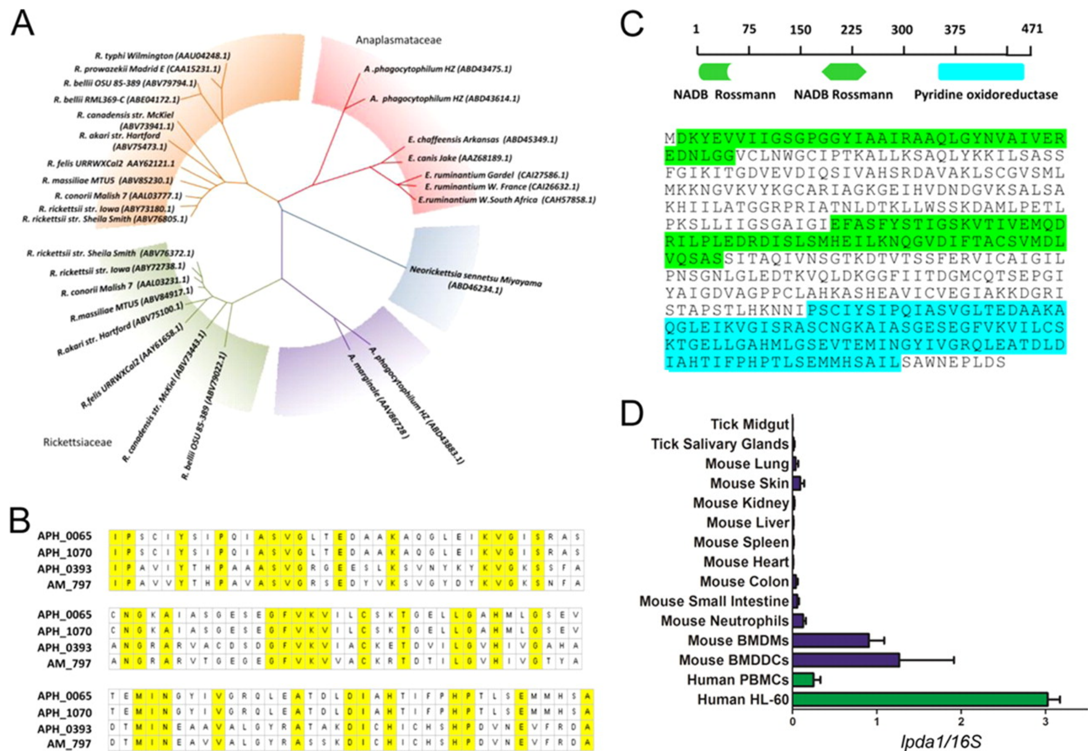

Figure 5: Characterization of the *A. phagocytophilum lpda1* gene. (A) Phenogram displaying similarities among many *lpda* genes from the families Anaplasmataceae and Rickettsiaceae. (B) Alignment of LPDA pyridine nucleotide-disulfide oxidoreductase dimerization domains from *A. phagocytophilum* (APH) and *A. marginale* (AM). Conserved regions are shown in yellow. (C) Diagram illustrating the LPDA1 domains. Data represent Rossmann-fold NAD $(P)^+$-binding proteins (NADB Rossmann; positions 2 to 40 and 185 to 243) and the pyridine nucleotide-disulfide oxidoreductase dimerization domain (Pyridine oxidoreductase; positions 352 to 461). (D) *Lpda1* transcription in cells or organs of *I. scapularis* ticks, mice, and humans measured by qRT-PCR and normalized to *A. phagocytophilum* infection (*lpda1*/*16S*). Data are presented as means ± SEM. BMDMs—bone marrow derived macrophages; BMDDCs—bone marrow derived dendritic cells; PBMC—peripheral blood mononuclear cells.

A. phagocytophilum LPDA1 affects host-induced immunopathology

To determine the *lpda1* function during *A. phagocytophilum* infection, an *A. phagocytophilum Himar1* transposon mutant library was constructed and screened using the clonal strain HZ (Figure 6A). Inverse PCR revealed a single transposon insertion into the *A. phagocytophilum* dihydrolipoamide dehydrogenase 1 (*lpda1 - APH_0065*) gene. By using two different doses (1x 10^7 or 1x 10^8 bacteria), no differences in pathogen load were detected when the *A. phagocytophilum lpda1::*TnHimar1 mutant was used to infect C57BL/6 mice (Figure 6B-C).

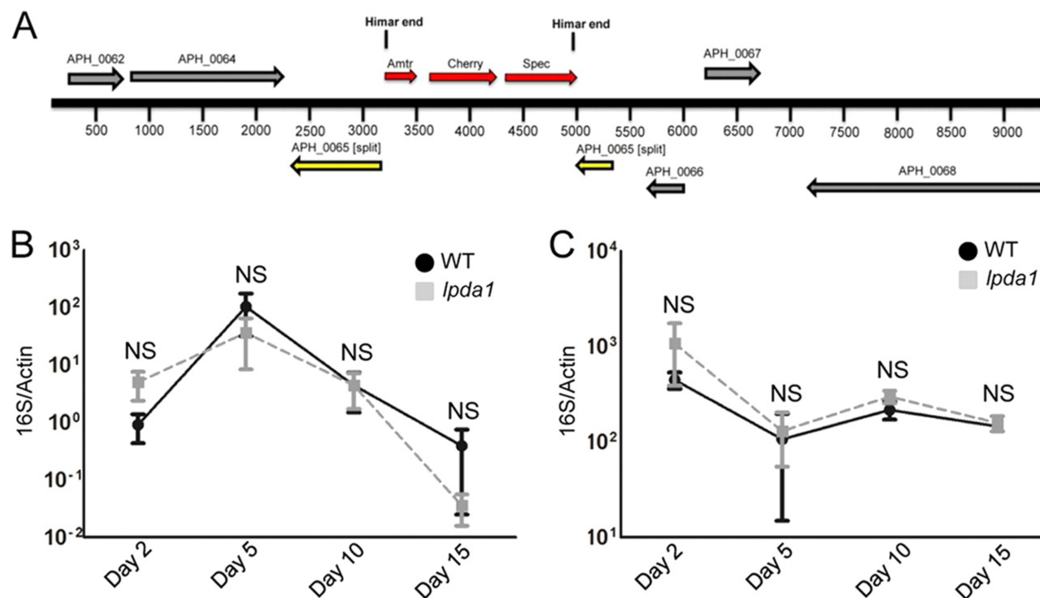

Figure 6: *A. phagocytophilum lpda1* does not affect bacterial load in mice. (A) Insertion diagram representing the location of the Himar1 transposon in the *A. phagocytophilum* HZ genome. The Himar 1 transposon was inserted into the *lpda1* gene (APH_0065), generating the *A. phagocytophilum* mutant *lpda1*::TnHimar1. *A. phagocytophilum* was transformed to express

the red fluorescence protein mCherry (Cherry) and the spectinomycin (Spec) antibiotic resistance gene under the control of the *amtr* promoter. The Himar end indicates transposon boundaries. (B and C) C57BL/6 mice were infected with the wild-type *A. phagocytophilum* strain HZ (WT) (n = 20) or the *lpda1*::TnHimar1 mutant (*lpda1*) (n = 20). Mice were infected by intraperitoneal injection with either (B) 10^7 or (C) 10^8 *A. phagocytophilum* bacteria, and bacterial load was measured by the absolute quantification qRT-PCR method at the indicated time points. Data represent the results of two-way ANOVA—Bonferroni (*lpda1*, WT); data are presented as means ± SEM. NS—not significant. $P < 0.05$.

Clinicopathological abnormalities, however, were observed when C57BL/6 mice were infected with the mutant *lpda1::*TnHimar1 strain at days 5 (Figure 7) and 15 post-infection (*data not shown*). Peripheral blood neutropenia and lymphopenia occurred in animals infected with the mutant *lpda1::*TnHimar1 when compared to naïve animals (Figure 7A and C). Mice infected with the mutant *lpda1::*TnHimar1 only had decreased neutropenia when compared to animals infected with the wild-type *A. phagocytophilum* HZ strain (Figure 7A). Thrombocytopenia (Figure 7B) and anemia (as judged by the hematocrit and red blood cell count) were only observed in mice infected with the wild-type *A. phagocytophilum* HZ strain when compared to naïve mice (Figure 7E-F). Anemia also occurred when mice infected with the wild-type *A. phagocytophilum* HZ strain were compared to animals infected the mutant *lpda1::*TnHimar1 strain. Both groups of infected mice had decreased total serum T4 when compared to non-infected mice with a more substantial decrease in wild-type bacterial infection (Figure 7G). Wild-type-infected

mice had decreased serum alkaline phosphatase (Figure 7H) and both groups of infected mice had decreased blood urea nitrogen (BUN) when compared to non-infected mice but no difference was observed between infected groups (Figure 7I).

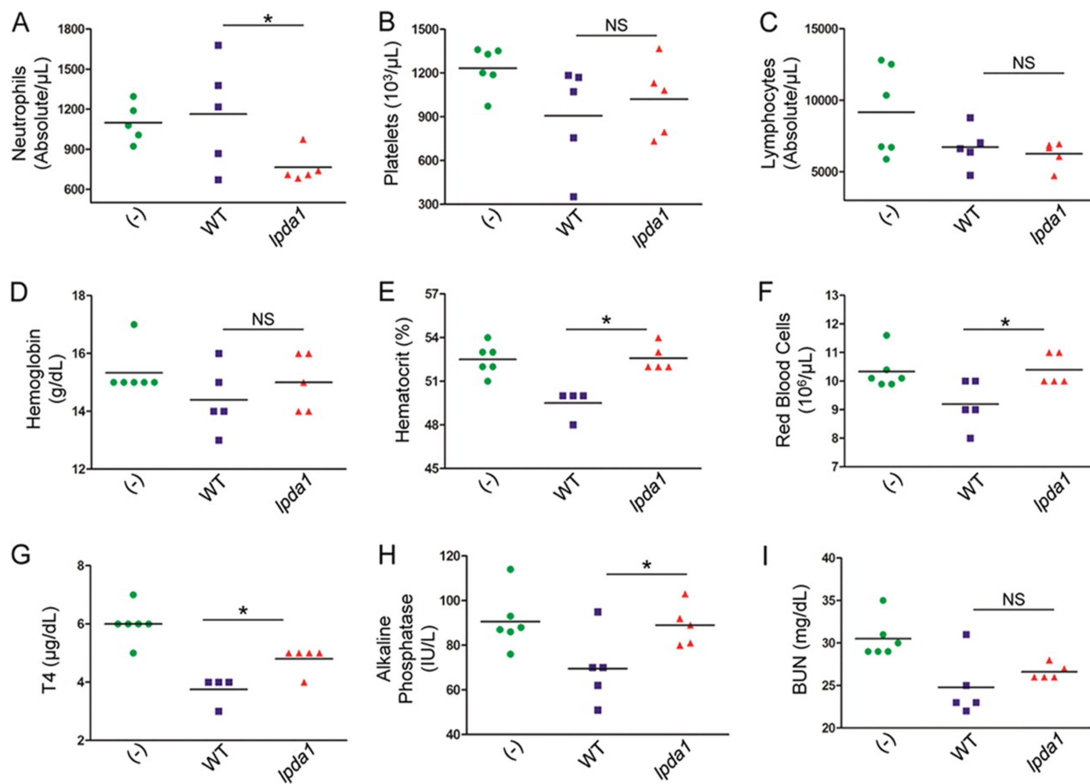

Figure 7: *A. phagocytophilum* LPDA1 causes haematological changes during pathogen infection of mice. C57BL/6 mice were infected with the wild-type *A. phagocytophilum* strain HZ (WT) (n = 4 to 6) or *lpda1*::TnHimar1 (n = 5) (*lpda1*) and contrasted to non-infected mice (-). Blood was collected at day 5 post-infection, and levels of (A) neutrophils, (B) platelets, (C) lymphocytes, (D) haemoglobin, (E) haematocrit, (F) red blood cells, (G) T4, (H) alkaline phosphatase, and (I) BUN were measured and results compared using an unpaired Student's t test (*lpda1*, WT). NS—not significant. Data represent means of the results. *, $P < 0.05$.

The mechanisms that drive hematological abnormalities during *A. phagocytophilum* mouse infection are not clearly understood. However, the myelosuppressive properties of interferon (IFN)-γ may be involved (MacNamara, Jones et al. 2011). Mice infected with the mutant *lpda1::*TnHimar1 strain had higher levels of IFN-γ in the peripheral blood when compared to wild-type-infected animals (*data not shown*). These findings correlated with increased spleen weight and splenic extra-medullary hematopoiesis in *lpda1*-infected mice when compared to wild-type-infected animals *(data not shown)*. Mice infected with the *A. phagocytophilum lpda1::TnHimar1* strain had peripheral neutropenia (Figure 7A) but no substantial increase in mature splenic neutrophils. On the other hand, mice infected with the wild- type *A. phagocytophilum* HZ strain had normal neutrophil counts in the peripheral blood but higher splenic neutrophilia (*data not shown*). Taken together, our findings suggest that LPDA1 affects the hematological profile and spleen hematopoiesis during *A. phagocytophilum* infection.

4.4 Concluding remarks

The immune response to *A. phagocytophilum* involves both innate and adaptive defenses. Innate immunity is involved in infection-induced immunopathology, whereas adaptive responses are responsible for eliminating *A. phagocytophilum* (Lepidi, Bunnell et al. 2000; Martin, Caspersen et al. 2001; Ehlers 2004; Scorpio, Von Loewenich et al. 2005; Browning, Garyu et al. 2006; Scorpio, von Loewenich et al. 2006; Choi, Webb et

al. 2007). Recognition of *A. phagocytophilum* and neutrophils' colonization is the first step for *A. phagocytophilum* establishment and propagation in the mammalian host. With the attempt to control infection, Polymorphonuclear cells respond to the bacterial threat by producing ROS. This, in turn, leads to tissue damage and host inflammation result in cytopenias and splenomegaly (Dumler, Madigan et al. 2007; Johns, Macnamara et al. 2009; Thomas, Dumler et al. 2009; Johns and Borjesson 2011), irrespective of bacterial load. Decreased bone marrow activity and liver dysfunction are also observed in HGA (Johns, Macnamara et al. 2009). The precise events and underlying molecules involved in *A. phagocytophilum* colonization of the mammalian host remain mostly unclear.

In this study, an *A. phagocytophilum* potential virulence molecule that regulates inflammation during pathogen infection was characterized. LPDA1 proved important to the disease state because mice infected with the *A. phagocytophilum* mutant *lpda1::TnHimar1* developed more pronounced splenomegaly when compared to the wild-type strain (*data not shown*). Splenomegaly is an overt clinical symptom in both mice and humans (Dumler, Madigan et al. 2007; Thomas, Dumler et al. 2009). Although not discussed here, the *lpda1* gene affected NF-κB activation leading to higher proinflammatory cytokine secretion during host anti-microbial response upon stimulation with the mutant pathogen, when compared to wild-type strain. The subversive role of a bacterial α-ketoacid dehydrogenase gene on NF-κB activation has not been previously described. This is the first report to establish a connection between LPDA1 and NF-κB in the context of microbial pathogenesis.

It is unclear why reducing cytokine secretion, and hence inflammation, would be important for *A. phagocytophilum* because inflammatory processes do not seem to have

a direct role in pathogen clearance. It is possible that control of inflammation via LPDA1 by *A. phagocytophilum* would shift hematopoietic progenitor and lineage-committed cells in the bone marrow. Inflammation-mediated shifts would increase the release of neutrophils and other cells that may serve as a host for pathogen colonization. This line of thinking is supported by recent studies showing that *A. phagocytophilum* causes hematopoietic alterations in the spleen with an expansion of lymphoid follicles and marked extra-medullary hematopoiesis in the red pulp (Johns, Macnamara et al. 2009; Johns and Borjesson 2011). Altered CXCL12/CXCR4 signaling – a critical pathway in hematopoietic and progenitor cell mobilization – also appear to be involved during *A. phagocytophilum* infection (Johns and Borjesson 2011).

Dihydrolipoamide dehydrogenase is a critical component of the α-ketoacid dehydrogenase intermediary metabolism in both prokaryotes and eukaryotes, and enables bacterial antioxidant defense (Bryk, Lima et al. 2002). This enzyme is a pyridine nucleotide:disulfide oxidoreductase that catalyzes flavin-dependent regeneration of the lipoamide cofactor (Bryk, Arango et al. 2010). The pathogenic role of dihydrolipoamide dehydrogenase in bacteria has been previously observed in *Streptococcus pneumonia* (Smith, Roche et al. 2002) and *Haemophilus influenza* (Deghmane, Soualhine et al. 2007) among others. Here, LPDA1 is described as conserved in the Anaplasmataceae and Rickettsiaceae families. The results provided here also demonstrate a direct role of *A. phagocytophilum* LPDA1 in infection-induced immunopathology. Future understanding of how components of the central carbon metabolism, such as LPDA1, contribute to *A. phagocytophilum* pathogenesis and disease will be critical for understanding *A. phagocytophilum* virulence and HGA etiology.

4.5 Materials and methods

Ethics Statement

Blood samples were obtained from healthy, non-pregnant adults and the Human Research Review Board at the University of California-Riverside approved this procedure. Animals were housed in the Animal Resources Facility according to the guidelines described under the federal Animal Welfare Regulations Act. Food and water were provided and the Institutional Animal Care and Use Committee at the University of California-Riverside approved all animal procedures. C57BL/6, mice were purchased from Jackson Laboratories. Mice at 6-10 weeks of age were used.

Bacterial strains

The University of California-Riverside approved the use of *A. phagocytophilum* strains. The *A. phagocytophilum* strains HZ and the mutant *lpda1::TnHimar1* were grown in HL-60 cells (ATCC CCL-240). Cells were maintained in IMDM with L-glutamine and HEPES (Hyclone, Thermo scientific), 20% heat-inactivated FBS in 5% CO2 and humidified air at 37°C. Cell lines were transformed to express mCherry and spectinomycin resistance under the control of the *amtr* promoter and flanked by the transposase recognition sequences using the Himar 1 transposon system. Construction of the transposase expression plasmid, bacterial transformation and selection and rescue cloning assays were performed as previously described (Felsheim, Herron et al. 2006). A single transposon insertion into the *A. phagocytophilum lpda1* sequence was

detected by inverse PCR. Sequence annotation was determined by using the genome browser software Artemis (Carver, Berriman et al. 2008).

Bioinformatics analysis

Dihydrolipoamide dehydrogenase domain structures were obtained from GenBank and the comprehensive microbial resource (CMR) genome database (http://cmr.jcvi.org). Phenogram of dihydrolipoamide dehydrogenase proteins was conducted using *MEGA* version 4.

RNA isolation and quantitative RT-PCR

Total RNA from organs, cells or tissues was isolated with TRIZOL reagent from Invitrogen. CDNA was prepared with the Verso cDNA kit (Thermo Scientific). Quantitative RT-PCR (qRT-PCR) was performed using the iQ SYBR Green Supermix and the Bio-Rad iQ5 Optical System. Measurement of *A. phagocytophilum* load was done as previously described (Pedra, Sutterwala et al. 2007). Primer sequences for *A. phagocytophilum* were*: 16s*-F: 5'-CAGCCACACTGGAACTGAGA-3' and *16s*-R: 5'-CCCTAAGGCCTTCCTCACTC-3'; *lpda1*F: 5’-ATCGTCAACAGTGGCACAAA–3’ and *lpda1*R: 5’ CTGATGTTTGGCACATACCG-3’.

Complete blood count and clinical chemistry

C57BL/6 mice were infected by intra-peritoneal injection with the *A. phagocytophilum* wild- type HZ or the mutant *lpda1::TnHimar1* strains ($1x10^8$ bacteria). Blood was collected at day 5 post-infection, and complete blood count and clinical chemistry analyses were performed in a commercial laboratory (Antech Diagnostics). Neutrophils, platelets, lymphocytes, hemoglobin, hematocrit, red blood cells, thyroxin (T4), alkaline phosphatase, and blood urea nitrogen (BUN) were measured.

Statistical analysis

Data were expressed as means standard errors of the means (SEM). Gaussian distribution was determined by the D'Agostino and Pearson normality test. For data points that followed a Gaussian distribution, the following parametric analyses were used: unpaired Student's t test (two-group comparisons) and analysis of variance (ANOVA) (comparisons of three or more groups; one or more variables). Bonferroni (parametric) post hoc multiple-comparison tests were used following ANOVA. All statistical calculations were performed by using GraphPad Prism version 5.04. Graphs were made by using GraphPad Prism version 5.04. P 0.05 was considered statistically significant.

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Chapter 5: Tick Saliva and *A. phagocytophilum*

5.1 Abstract

Ticks use saliva as a deference strategy against host immunity. Prolonged feeding of ticks enables transmission of several infectious agents to the mammalian host. While the extraordinary anti-inflammatory properties of tick saliva have been previously demonstrated, how *I. scapularis* saliva influences cytokine secretion and host colonization by the obligate intracellular rickettsial pathogen *A. phagocytophilum* remains mostly elusive. Here, *I. scapularis* saliva is shown to inhibit cytokine secretion by mammalian immune cells during challenge with *A. phagocytophilum*. More specifically, the tick salivary protein sialostatin L2 reduces inflammation *in vivo* through caspase-1 in the presence of *A. phagocytophilum.* Taken together, these findings result in a new understanding of *A. phagocytophilum* pathogenesis and immunity and suggest that *I. scapularis* may have evolved strategies for modulating host defenses during rickettsial transmission.

5.2 Introduction

Hematophagy seems to have evolved independently in more than 14,000 arthropod species. Alongside, several complementary biological adaptations have also occurred. Salivary gland secretion is among the most common adaptations in hematophagous arthropods, and proteins from ticks, mosquitoes, biting flies, fleas and other blood-feeding arthropods promote defense against host homeostasis and inflammation (Paesen, Adams et al. 2000; Valenzuela 2004; Hovius, Levi et al. 2008; Francischetti, Sa-Nunes et al. 2009; Andersen 2010; Dai, Narasimhan et al. 2010; Fontaine, Diouf et al. 2011; Chmelar, Calvo et al. 2012). By blood feeding, however, arthropods have also become capable of acquiring microorganisms found in the host blood, and hence, many infectious diseases are transmitted by hematophagous arthropods.

Interestingly, researchers have described that saliva facilitates the establishment of vector-borne pathogens (Fontaine, Diouf et al. 2011). This phenomenon was first seen during infection by *Leishmania* parasites (Titus and Ribeiro 1988), and following studies demonstrated that enhanced pathogen transmission by saliva is universal among blood-feeding arthropods (Francischetti, Sa-Nunes et al. 2009; Fontaine, Diouf et al. 2011). For instance, mosquito saliva augments the transmission of malaria parasites (Vaughan, Scheller et al. 1999), West Nile (Styer, Lim et al. 2011), La Crosse (Osorio, Godsey et al. 1996) and Cache Valley viruses (Edwards, Higgs et al. 1998). Likewise, tick saliva counteracts host-derived inflammation (Francischetti, Sa-Nunes et al. 2009; Fontaine, Diouf et al. 2011) by impairing the function of innate and adaptive immune cells (de Silva, Tyson et al. 2009), and inhibiting cytokine secretion (Fontaine, Diouf et

al. 2011). It also promotes survival of the Lyme disease agent, *B. burgdorferi*. This spirochete seems to be shield by a salivary protein called salp15 from the tick *I. scapularis*, and in turn, protected from antibody-mediated killing (Ramamoorthi, Narasimhan et al. 2005) and dendritic cell function (Hovius, de Jong et al. 2008).

A. phagocytophilum is a pathogen transmitted by ixodid ticks and causes HGA, an emerging infectious disease in the United States, Europe and Asia (Severo et al., 2012). Macrophages were recently shown to be important for defense against *A. phagocytophilum* colonization (Chen, Severo et al., 2012a and b). These phagocytic cells are essential to the regulation of immune responses and inflammation. Nevertheless, it remains mostly unknown whether tick saliva affects macrophages' function during stimulation with *A. phagocytophilum*. This chapter illustrates that tick saliva mitigates *A. phagocytophilum*-induced cytokine secretion by murine macrophages. Moreover, it shows that a specific salivary protein - sialostatin L2 - from the tick *I. scapularis* inhibits inflammasome activity during *A. phagocytophilum* stimulation. The inflammasome, a multi-protein complex necessary for IL-1β maturation, is critical for the inflammatory process (Strowig, Henao-Mejia et al. 2012). This is the first report of a salivary protein from a disease vector inhibiting inflammasome function during pathogen transmission.

5.3 Results

I. scapularis saliva inhibits IL-1β secretion by macrophages during *A. phagocytophilum* stimulation

A dogma has emerged in the last few years in which the production and release of IL-1β are the result of a two-tier system: one signal is induced by pattern recognition receptors or pro-inflammatory cytokines to activate the transcription and translation of IL-1β via NF-κB. This signal is also referred as priming and it is typically done by LPS stimulation of immune cells. The second signal is mediated by the inflammasome, a protein scaffold, to induce the cleavage of IL-1β into its mature form through caspase-1 activation (Latz ; Schroder, Zhou et al. ; Tiemi Shio, Eisenbarth et al. 2009). Our studies show that IL-1β secretion triggered by *A. phagocytophilum* during bone marrow derived macrophages (BMDMs) stimulation does not require LPS priming (Figure 8A). To the contrary, LPS priming in BMDMs before *A. phagocytophilum* stimulation inhibited IL-1β secretion (*data not shown*). These results are consistent with the lack of genes for LPS and peptidoglycan synthesis in the *A. phagocytophilum* genome (Hotopp, Lin et al. 2006) and suggest that molecules other than LPS may prime BMDMs for production of pro-IL-1β. Further, tick saliva inhibited *A. phagocytophilum*-induced IL-1β secretion by BMDMs (Figure 8A). IL-1β secretion may be also coupled to cell death (Strowig, Henao-Mejia et al. 2012), but only low levels of cell death during *A. phagocytophilum* stimulation of BMDMs were observed in the absence of tick saliva (~10-15%). Nevertheless, tick saliva was able to mitigate the effect of pathogen stimulation on cell death to background levels (~5-8%) (Figure 8B).

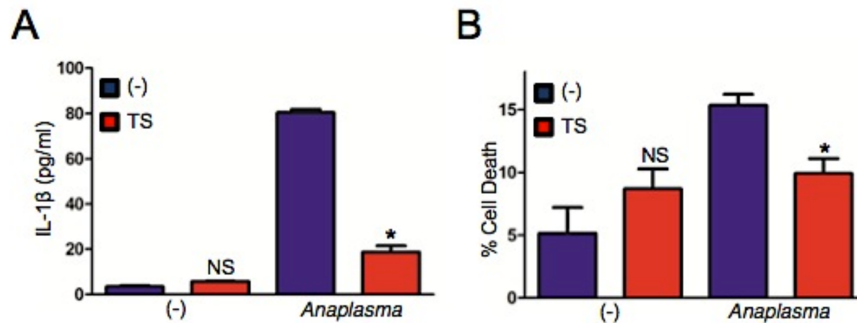

Figure 8: *I. scapularis* saliva decreases IL-1β secretion by macrophages during *A. phagocytophilum* stimulation. (A) BMDMs ($1{\times}10^6$) from C57BL/6 mice were stimulated with the wild-type *A. phagocytophilum* HZ strain (MOI 50) for 18 hours in the presence or absence of tick saliva (1:150 dilution). IL-1β was measured by ELISA. (B) Cell death was measured using the LDH assay. Responses were measured in triplicate and presented as mean ± SEM within the representative experiment. *, $P < 0.05$, Student's t test. (−) non-stimulated cells. NS – not significant.

Sialostatin L2 inhibits IL-1β-induced inflammation by A. phagocytophilum in vivo

The cysteine protease inhibitor (cystatin) sialostatin L2 is a tick salivary protein previously shown to be immunomodulatory (Kotsyfakis, Karim et al. 2007; Kotsyfakis, Anderson et al. 2008; Kotsyfakis, Horka et al. 2010). Macrophages are the main producers of IL-1β, an inflammasome-derived cytokine, and inflammasome activation is important for *A. phagocytophilum* immunity. Thus, the effect of sialostatin L2 in IL-1β

secretion by BMDMs during *A. phagocytophilum* stimulation was evaluated. The results showed that sialostatin L2 inhibited IL-1β secretion during *A. phagocytophilum* stimulation of BMDMs at MOIs 10 and 50 *(data not shown).*

To determine whether sialostatin L2 could inhibit the inflammasome *in vivo,* intradermal injections of sialostatin L2 were performed in the presence of *A. phagocytophilum*. Intradermal injection of mice was the preferred method to perform *in vitro* studies because RNAi silencing of sialostatin L2 in ticks impairs feeding (Kotsyfakis, Anderson et al. 2008) and a reliable comparison of pathogen transmission in control and RNAi-silenced ticks is not possible. Similar to the positive control LPS, intradermal injection of *A. phagocytophilum* in mice led to caspase-1 activation (p20) and IL-1β maturation (Figure 9). Consistent with our results observed for BMDMs, sialostatin L2 impaired caspase-1 activation and IL-1β maturation triggered by *A. phagocytophilum* at the skin site (Figure 9). Furthermore, IL-1β translation was not affected by sialostatin L2 *in vivo*, suggesting that NF-κB signaling is not affected by sialostatin L2 in the *A. phagocytophilum* infectious disease model (Figure 9).

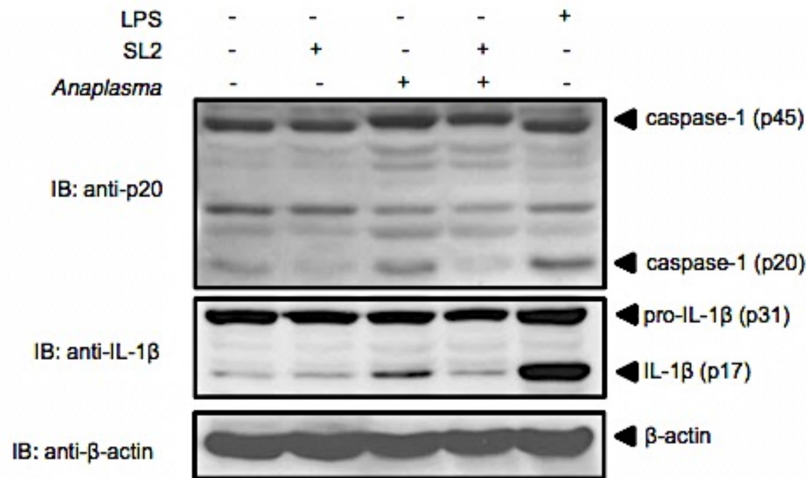

Figure 9: Sialostatin L2 inhibits caspase-1 activation and IL-1β maturation during *A. phagocytophilum* infection *in vivo*. Intradermal injection of C57BL/6 mice with sialostatin L2 (SL2) (20 μg), *A. phagocytophilum* (1 x10^4), SL2 (20 μg) + *A. phagocytophilum* (1 x10^4) and LPS (40 μg). Proteins from the injection site were extracted and western blot (IB) was performed to detect caspase-1 (p45 and p20) and IL-1β (p31 and p17). β-actin was used as a loading control.

Next, it was determined whether sialostatin L2 would affect inflammation at the skin site (as judged by neutrophil and macrophage infiltration). The results demonstrate that, similar to LPS, *A. phagocytophilum* induces neutrophil infiltration within the dermis, subcutaneous adipose tissue and skeletal muscle of host skin (Figure 10).

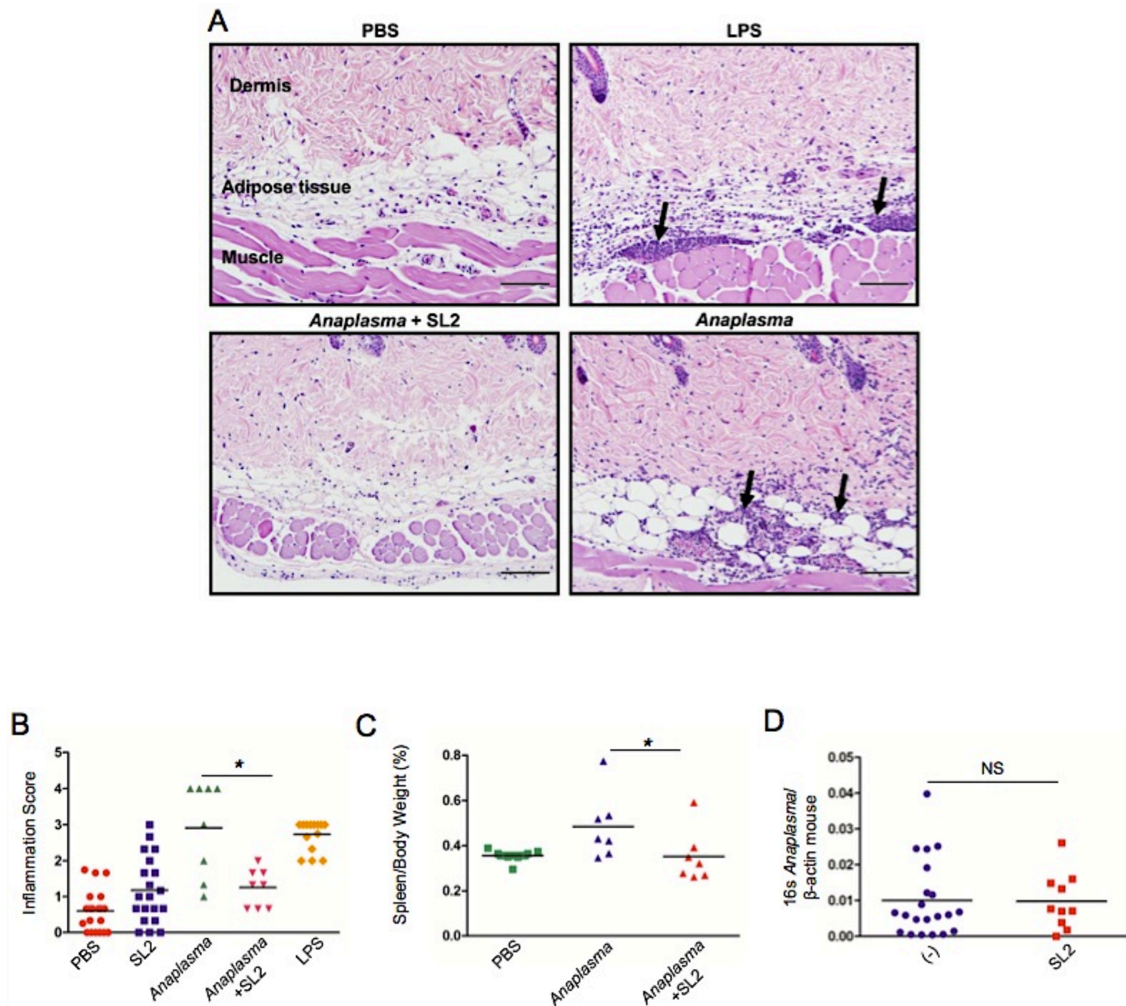

Figure 10: Sialostatin L2 inhibits *A. phagocytophilum*-induced inflammation at the skin site. Intradermal injection of C57BL/6 mice with PBS (-), sialostatin L2 (SL2) (20 μg), *A. phagocytophilum* (1 x10^4), SL2 (20 μg) + *A. phagocytophilum* (1 x10^4) and LPS (40 μg). (A) Skin inflammation characterized by infiltration of neutrophils, eosinophils and few macrophages in the dermis, subcutaneous adipose tissue and underlying skeletal muscle of mice (arrows). H&E staining at 200X magnification. Bar=100 µm. (B) Inflammation score, as described in the materials and methods. (C) Spleen weight of C57BL/6 mice infected with *A. phagocytophilum* (n=7) and SL2 (20 μg) + *A. phagocytophilum* (n=7) normalized to the animal's body weight and

contrasted to non-infected mice (n=7) (PBS) at day 14 post-intraperitoneal infection. (D) Bacterial load following intradermal infection of *A. phagocytophilum* in the presence of SL2 (10 µg/injection). SiL2 was injected 5 hours prior, during and 5 hours post-pathogen inoculation. Bacterial load was measured 24 hours post-injection by qRT-PCR. *, $P < 0.05$, (B) Kruskal-Wallis (post-hoc Dunn's). (C) ANOVA (post-hoc Bonferroni) (D) Student's t test. NS – not significant.

Importantly, sialostatin L2 alone did not stimulate cell infiltration at the skin (Figure 10C). Moreover, sialostatin L2 inhibited inflammation at the skin site during *A. phagocytophilum* infection. Injection of sialostatin L2 together with *A. phagocytophilum* reduced inflammation to background levels (PBS panel) (Figure 10). The anti-inflammatory effect of sialostatin L2 during *A. phagocytophilum* injection was not due to direct binding of sialostatin L2 to *A. phagocytophilum.* Our *in vitro* binding assays did not show any direct interactions between *A. phagocytophilum* and sialostatin L2 (*data not shown*).

5.4 Concluding remarks

Inflammation is characterized by complex interactions between innate and adaptive immunity (Newton and Dixit 2012). Pro-inflammatory cytokines and chemokines recruit immune cells to the site of tick feeding. Tick salivary proteins then mitigate the secretion of cytokines by immune cells, therefore diminishing inflammation

(Francischetti, Sa-Nunes et al. 2009; Fontaine, Diouf et al. 2011; Chmelar, Calvo et al. 2012). Despite significant progress in the past decades, how ectoparasites, such as ticks, regulate innate immune signaling during transmission of the rickettsial agent *A. phagocytophilum* to the mammalian host remains mostly elusive. In this chapter, *I. scapularis* saliva was shown to have the ability to inhibit cytokine secretion mediated by murine immune cells during stimulation with the rickettsial pathogen *A. phagocytophilum*. More specifically, the tick salivary protein sialostatin L2 inhibited inflammasome-mediated inflammation upon infection with *A. phagocytophilum*. The sialostatin L2 effect on caspase-1 activation and IL-1β secretion appeared specific for *A. phagocytophilum* because stimulation of macrophages with either *P. aeruginosa* (a non-vector borne pathogen) or *F. tularensis* (a non-*I. scapularis* tick pathogen) was not affected (*data not shown*). This is not entirely surprising because the intricate relationship between the tick vector and *A. phagocytophilum* is molded by evolutionary selection (Fontaine, Diouf et al. 2011).

One may consider the effect of sialostatin L2 on inflammasome biology during tick infestation. These experiments could not be performed because they are technically challenging. First, RNAi silencing and vaccination against sialostatin L2 impairs the feeding ability of *I. scapularis* nymphs (Kotsyfakis, Anderson et al. 2008; Kotsyfakis, Horka et al. 2010). Therefore, a reliable comparison of pathogen transmission in control and RNAi-silenced ticks; or, alternatively immunized and control groups is not possible. Second, mice are natural hosts of ticks; thus, they do not typically develop immunity against salivary proteins (Francischetti, Sa-Nunes et al. 2009; Chmelar, Calvo et al. 2012). Third, many sialostatin L2 paralogues are present in the *I. scapularis* genome. Hence, these molecules may cross-react with antibodies, questioning the validity of any

assay that measures sialostatin L2 concentration in the tick saliva. Due to these shortcomings, and the fact that potential host receptors (or cell mediators) in immune cells need saturation to reveal a noticeable phenotype, our approach of 'decomposing' tick saliva by studying individual molecules may be regarded as a conceptual advance for the understanding of inflammasome biology and vector-borne diseases.

Sialostatin L2 may not be the only vector salivary protein that inhibits inflammasome activation and regulates inflammation during pathogen infection. Biologically active proteins in the tick saliva are commonly used as a strategy for immune evasion during feeding (Francischetti, Sa-Nunes et al. 2009; Fontaine, Diouf et al. 2011). Ticks also have large genomes and carry many gene paralogs (Pagel Van Zee, Geraci et al. 2007). These gene paralogs may act redundantly to provide inhibition of protein scaffolds, such as the inflammasome, in the mammalian host. Earlier studies provide experimental support for this scenario. Ramachandra and Wikel showed that salivary gland extracts from the tick *Dermacentor andersoni* reduced IL-1 levels during the early phases of tick feeding (Ramachandra and Wikel 1992), while another group determined that human IL-1β secretion was mitigated when treated with LPS and salivary gland extracts from partially fed adult female *Rhipicephalus appendiculatus* ticks (Fuchsberger, Kita et al. 1995).

Currently, vaccines for arthropod-borne diseases are only available for the yellow fever virus, Japanese encephalitis virus, Rift valley fever virus and the tick-borne encephalitis virus (Fontaine, Diouf et al. 2011). The association of traditional pathogen- and vector-based vaccines could improve protection against vector-borne diseases. This rationale is further supported by work showing that previous exposure of mice to salivary

gland extracts of sandflies, mosquitoes and ticks reduce pathogen load and vector fitness during transmission (Francischetti, Sa-Nunes et al. 2009; Fontaine, Diouf et al. 2011). The effective use of salivary gland molecules that target the inflammasome as vaccine candidates could, in theory, be, used to reduce morbidity and mortality associated with major vector-borne diseases.

In conclusion, this study shows *I. scapularis* saliva inhibits cytokine secretion mediated by macrophages infected with *A. phagocytophilum*. It also demonstrates that the *I. scapularis* tick salivary protein sialostatin L2 reduces inflammasome activation and dampens inflammation in the mouse skin during *A. phagocytophilum* inoculation. Elucidating the mechanisms by which sialostatin L2 inhibits inflammasome assembly during *A. phagocytophilum* transmission may have direct implications in our understanding of how ticks circumvent defenses promoted by the mammalian immune system during feeding. The implications for these findings are wide in scope as ticks, mosquitoes, biting flies, fleas and blood feeding bugs have also evolved similar strategies for modulating host defenses and pathogen transmission (Fontaine, Diouf et al. 2011).

5.5 Materials and methods

Ethical statements

The Institutional Animal Care and Use Committee at the University of California-Riverside approved all animal experiments. Mice at 6-10 weeks of age were used.

C57BL/6 was purchased from Jackson Laboratories. Experimentation with *A. phagocytophilum* (HZ strain) was approved by the Biological Use Authorization (BUA) Committee at the University of California-Riverside.

Saliva and Sialostatin Preparation

To isolate vector saliva*, I. scapularis* ticks were allowed to feed on New Zealand white rabbits. A restraining collar was placed around the neck of each rabbit, and their ears were covered prior to tick exposure. Ticks were permitted to engorge for 4–5 days on the ear of a rabbit. Upon harvesting, ticks were rinsed in distilled water and were immediately fixed to glass slides with double-sided tape. A sterile glass micropipette was placed around the hypostome to collect saliva. Salivation was induced by the application of pilocarpine to the scutum of the tick. Saliva was pooled and stored at −80°C for use.

Sialostatin L2 was produced, as previously described (Kotsyfakis, Karim et al. 2007; Kotsyfakis, Anderson et al. 2008; Kotsyfakis, Horka et al. 2010). Briefly, sialostatin L2 cDNA was PCR-amplified and subcloned into the pET17b bacterial expression vector. The expression vector was placed into the *Escherichia coli* strain BL21(DE3)pLysS for expression. Cultures were grown and induced by adding isopropyl 1-thio-β-d-galactopyranoside (IPTG). Inclusion bodies were dissolved in 6 M guanidine hydrochloride, 20 mm Tris-HCl, pH 8.0, and reduced with 10 mM dithiothreitol (DTT). Sialostatin L2 was refolded in a large volume of 20 mM Tris HCl, pH 8.0, 300 mM NaCl, and stirred overnight at 4 °C. The refolded protein was concentrated with a tangential flow filtration device and purified by gel filtration chromatography on Sephacryl S-100

followed by anion exchange chromatography on Q-Sepharose. Dialysis followed against 20 mM Tris-HCl, pH 7.4, 150 mm NaCl. LPS contamination was removed by using the detergent-based method from Arvys Proteins. Endotoxin presence was estimated by using a sensitive fluorescence-based endotoxin assay from Lonza Biologics.

Cell Culture Generation and Stimulation

Bone marrow-derived macrophages (BMDMs) were generated as previously described with minor modifications (Johnson, Kitz et al. 1983). Briefly, femurs and tibias were removed from mice and kept in phosphate buffered saline (PBS) + 1% Penicillin-Streptomycin-Amphotericin (PSA) (ThermoScientific). Muscle was removed from femurs and tibias using scissors and razor blades. The ends were cut and marrow was flushed from the bone using cold Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) with a 27 gauge needle. BMDMs were grown on 10 cm petri dishes in 10 ml of DMEM media supplemented with 10% fetal calf serum (FCS) (Invitrogen), 30% L929 cell conditioning medium, and 1% PSA. BMDMs were grown in a humidified incubator at 37°C with 5% CO_2 for 6 days prior to stimulation. On the 3rd day, 10 ml of DMEM + 10 % FCS + 30% L929 cell conditioning medium + 1% PSA was added to each dish. BMDMs were plated on 24-well culture plates at 1 x 10^6 cells per well, unless otherwise stated, in 500 µl of DMEM + 10% FCS + 1% PSA. Chemical inhibitors or sialostatin L2 were added at indicated concentrations and prior to *A. phagocytophilum* stimulation. BMDMs were also stimulated with LPS. Cell death was measured by using the lactate dehydrogenase (LDH) kit (Roche).

Immunoblotting

Cell lysates were extracted using radioimmunoprecipitation (RIPA) lysis buffer (Boston Bioproducts) with Complete Mini Protease Inhibitor Cocktail and PhosSTOP, both from Roche Applied Science. Protein concentration was determined via the Bradford protein assay method, using protein assay dye reagent concentrate and iMark reader, both from Bio-Rad. SDS polyacrylamide gel was made and ran at 200 volts for 1 hour. Transfer was done in wet conditions with polyvinylidene fluoride (PVDF) membranes for 90 minutes at 100 volts. Membranes were blocked in 5% non-fat dry milk (LabScientific, Inc.). Western blot antibodies for β-actin (Neomarker-Thermo Scientific) (1:500-1:1000) (Catalogue number - RB-9421p), IL-1β (R&D systems) (1:1000) (Catalogue number - AF-401-NA), caspase-1 (Millipore) (1:1000) (Catalogue number – 06-503), (Santa Cruz) (1:100-1:1000) (Catalogue number – SC-514). In some experiments, supernatants were concentrated with centrifugal filter units (3K) (Amicon) (Catalogue number – UFC500324) and caspase-1 immunoblots were performed. Enhanced chemiluminescence (ECL) western blotting substrate and super signal West Pico Chemiluminescent substrate were used to image the blots (Pierce Thermo Scientific). Densitometry was performed for immunoblot band intensity quantification using ImageJ (http://rsbweb.nih.gov/ij/).

Enzyme-Linked Immunosorbent Assay (ELISA)

Mouse IL-1β was measured with the BD OptEIA Set from BD Biosciences. Supernatants were collected and absorbance was measured using Bio-Rad iMark at 450 nm with a 595 nm correction.

***In vivo* infection**

To study the effects of sialostatin L2 on local inflammation, back-shaved C57BL/6 mice received intradermal injections with the following: 1) 20 µl of phosphate buffered saline (PBS), 2) sialostatin L2 (20 µg) (Invivogen), 3) *A. phagocytophilum* Hz strain (1 x10^4), 4) sialostatin L2 + *A. phagocytophilum* Hz strain, and 5) LPS (40 µg) on separate sites of dorsal skin. Sialostatin L2 was injected 5 hours prior*,* at the start and 5 hours post-bacterial infection. The skin surrounding injected sites was excised from each mouse 24 hours after treatment. The skin was fixed with 10% neutral buffered formalin for histopathologic study. Measurement of *A. phagocytophilum* load was done by using quantitative RT-PCR, as previously described (Pedra, Sutterwala et al. 2007). Primer sequences for *A. phagocytophilum* were*: 16s*F: 5'-GTTCGGAATTATTGGGCGTA- 3' and *16s*R-5'-GGAATTCCGCTATCCTCTCC-3'.

Histopathology

Formalin-fixed skin was sectioned longitudinally and stained with hematoxylin and eosin. A pathologist blinded to the groups scored sections for inflammation and determined the degree of inflammation. Four skin sections from each animal per treatment were evaluated and scored for dermal and subcutaneous adipose/skeletal muscle inflammation. Subcutaneous adipose/skeletal muscle inflammation was graded on a scale (0-3) based on granulocyte number; 0 (<5), 1 (5-10), 2 (10-50), 3 (>50). The scores from 3, 400X fields were averaged to determine subcutaneous adipose/skeletal muscle score per tissue section. Dermal inflammation was scored as either absent (0) or present (1). The scores were added to determine the skin inflammation score.

Statistical analysis

Data were expressed as means ± standard errors of the means (SEM). The following parametric analyses were used: unpaired Student's *t* test (two-group comparisons); one-way analysis of variance (ANOVA) (comparisons of three or more groups); Bonferroni or Kruskal-Wallis post hoc multiple-comparison tests were used following ANOVA. All statistical calculations were performed by using GraphPad Prism version 5.04. Graphs were made by using GraphPad Prism version 5.04. $P < 0.05$ was considered statistically significant.

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Chapter 6: The Tick XIAP and *A. phagocytophilum*

6.1 Abstract

Ubiquitination is a posttranslational modification that regulates protein degradation and signaling in eukaryotes. While it is acknowledged that pathogens exploit ubiquitination to infect mammalian cells, it remains unknown how microbes interact with the ubiquitination machinery in medically-relevant arthropods. This chapter highlights that the ubiquitination machinery is present in the tick *I. scapularis* and demonstrate that the E3 ubiquitin ligase named x-linked inhibitor of apoptosis protein (XIAP) restricts bacterial colonization of this arthropod vector. It also provide evidences that *xiap* silencing significantly increases tick colonization by the bacterium *A. phagocytophilum* - the causative agent of human granulocytic anaplasmosis. Here, it is also demonstrated that: i) XIAP polyubiquitination is dependent on the really interesting new gene (RING) catalytic domain; ii) XIAP polyubiquitination occurs via lysine (K) 63 but not K48 residues; and iii) XIAP-dependent K63 polyubiquitination requires zinc for catalysis. Taken together, these data define a role for ubiquitination during bacterial colonization of medically relevant arthropods.

6.2 Introduction

Ubiquitin is an evolutionarily conserved protein that carries seven lysine (K) amino acids (K6, K11, K27, K29, K33, K48, and K63). Ubiquitin may form linkages with the K of a target protein or the K of another ubiquitin (Welchman, Gordon et al. 2005; Skaug, Jiang et al. 2009). This process is referred to as protein ubiquitination and is employed to the control of numerous cellular processes. In fact, ubiquitination plays a central role in signaling networks that activate transcription factors like the NF- κB family, and has emerged as a key mechanism regulating pathogenesis and immunity in mammals. Hence, pathogens have evolved to exploit the ubiquitination machinery as an immune evasion mechanism, such as by targeting host proteins to degradation via the ubiquitin-proteasome system and reversing protein ubiquitination to inhibit defense responses (reviewed in (Jiang and Chen 2011)).

Ubiquitination involves a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin-protein ligase (E3) (Welchman, Gordon et al. 2005; Skaug, Jiang et al. 2009). Medically-relevant arthropod vectors have not been studied in the context of ubiquitination, and ubiquitin dynamics during pathogen colonization has yet to be explored. This is a scientific constraint because understanding the ubiquitination machinery in disease vectors may pave the ground for the development of novel therapeutics that prevent or delay the onset of illnesses.

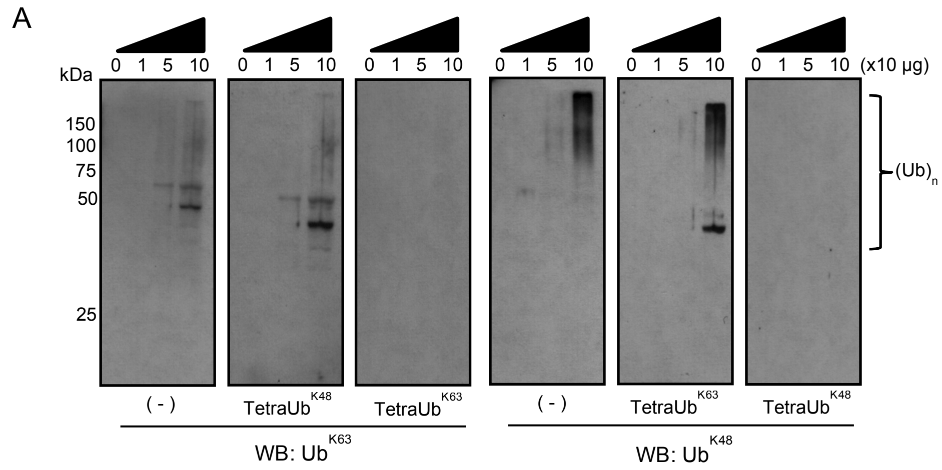
Here, how ubiquitination regulates microbial pathogenesis begins in ticks to be unraveled. By using the blacklegged tick and the rickettsial bacterium *A. phagocytophilum* (Severo, Stephens et al. 2012), the ubiquitome is first shown to be functional in *I. scapularis*. Then, the tick E3 ubiquitin ligase named x-linked inhibitor of

apoptosis protein (XIAP) is characterized via biochemical and molecular assays. XIAP polyubiquitination is described as dependent on the really interesting new gene (RING) domain, requires zinc for catalysis and occurs via lysine (K) 63 but not K48 residues. Finally, RNA interference (RNAi) approaches show that *xiap* silencing significantly increases colonization of *I. scapularis* ticks by *A. phagocytophilum*. Altogether, these findings shed some light onto microbial colonization of ticks and may serve as a prelude for discussions in terms of ubiquitin-pathogen interactions in disease vectors.

6.3 Results

The I. scapularis ubiquitome

K48 (Ub^{K48}) and K63 (Ub^{K63})-ubiquitination are the most widely studied ubiquitin chains (Newton, Matsumoto et al. 2008). Thus, antibodies specific for these linkages were used to determine whether Ub^{K48}- and/or Ub^{K63}-polyubiquitination are present in *I. scapularis*. Both Ub^{K48} and Ub^{K63} linkages were found to be present in protein lysates of the ISE6 cell line (Figure 11A, left panels). To confirm specificity, antibody-competition assays with linkage-specific tetraubiquitins were performed. $TetraUb^{K63}$ and $tetraUb^{K48}$ are the minimum recognition units by the polyubiquitin antibodies (Newton, Matsumoto et al. 2008). Co-incubation of Ub^{K63}- or Ub^{K48}-specific antibodies with their respective tetraubiquitin units abolished recognition of polyubiquitination (Figure 11A, right panels). Conversely, $tetraUb^{K48}$ co-incubated with the Ub^{K63}-specific antibody or $tetraUb^{K63}$ co-incubated with the Ub^{K48}-specific antibody did not affect polyubiquitination recognition (Figure 11A, center panels).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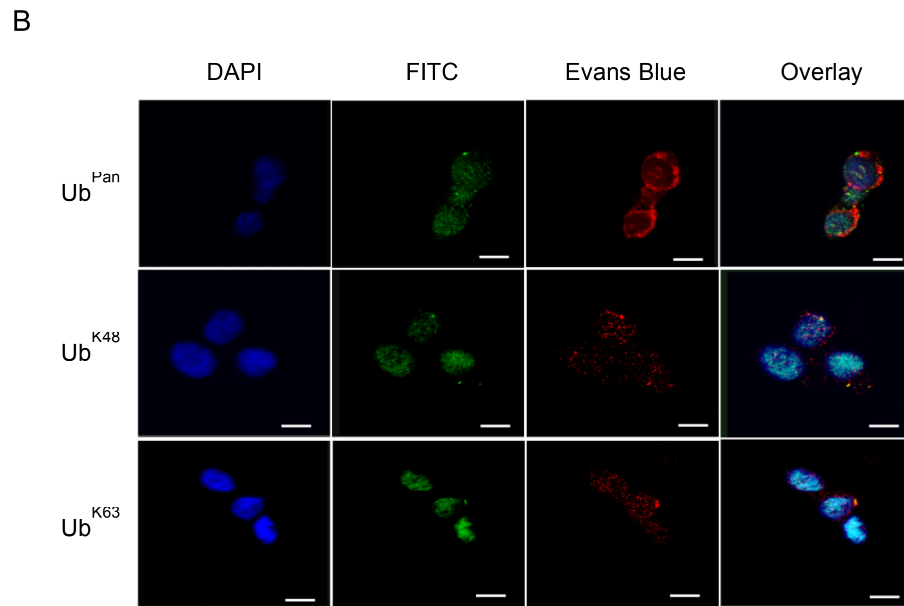
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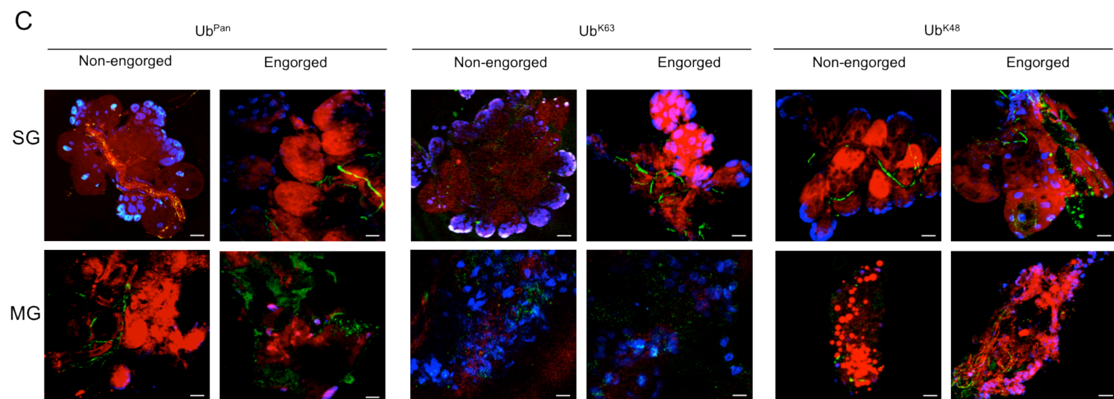


Figure 11: Polyubiquitination in *I. scapularis*. (A) Protein lysates were obtained from *I. scapularis* ISE6 cells and aliquots (10-100 µg) resolved in 10% SDS-PAGE. (-) represent immunoblots treated with Ub^{K63} and Ub^{K48} antibodies. Antibody specificity was assessed by pre-incubating the antibodies with either $TetraUb^{K48}$ or $TetraUb^{K63}$ for 1 hour prior to immunoblotting. (B) ISE6 cells, (C) tick salivary glands (SG) and midguts (MG) were fixed with paraformaldehyde and stained with DAPI (blue), Evans blue (red) and Ub^{Pan}, Ub^{K63} or Ub^{K48} antibodies (FITC). The scale represents 10 µm in (B) and 20 µm in (C). Original magnification at 63X (B) and 20X (C). These experiments were repeated twice.

Confocal microscopy with an antibody that recognizes a wide range of ubiquitin chains, here described as a pan ubiquitin (Ub^{Pan}) antibody, showed wide polyubiquitination distribution across ISE6 cells (Figure 11B, upper panel). However, foci of Ub^{K48} polyubiquitination in ISE6 cells revealed a pattern within the nuclear and perinuclear cellular region (Figure 11B, middle panel). Seemingly denser, Ub^{K63} polyubiquitination foci patterns were observed in ISE6 cells (Figure 11B, lower panel).

Since ticks experience a dramatic change in physiology during blood feeding (Piesman and Eisen 2008), polyubiquitination *in vivo* was addressed. The studies focused on salivary glands and midguts of non-engorged and engorged ticks because these organs are targeted by pathogens during a blood meal (Bowman and Sauer 2004; Piesman and Eisen 2008). It was difficult to estimate the extent to which differences observed were due to feeding or tissue reorganization because engorgement affected Ub^{Pan}, Ub^{K63} and Ub^{K48} polyubiquitination dynamics in tick midguts and salivary glands (Figure 11C). As in ISE6 cells, Ub^{K63} polyubiquitination was present in the nucleus of non-engorged tick salivary glands (Figure 11C, SG non-engorged middle panel). Conversely, no Ub^{K48} polyubiquitination in the nuclei of non-engorged tick salivary glands was detected (Figure 11C, SG non-engorged right panel). A more widespread distribution of Ub^{K48} polyubiquitination was seen after tick engorgement in both salivary glands and midguts (Figure 11C, engorged right panels). Less Ub^{K63} ubiquitination was observed in blood-fed midguts when compared to Ub^{K48} and Ub^{Pan}, but it is unclear if this effect is due to engorgement or blood residual. Nevertheless, Ub^{K63} can still be seen in the nuclear area of the midgut cells (Figure 11C, please note turquoise). Overall, these data support a functional ubiquitome in *I. scapularis* ticks.

I. scapularis XIAP is an E3 ubiquitin ligase

The human XIAP is an important E3 ubiquitin ligase involved in neutrophil infection by the tick-borne rickettsial agent *A. phagocytophilum* (Ge and Rikihisa 2006). The tick XIAP sequence suggested similarities with mammalian XIAPs and related proteins in *Drosophila* (Figure 12A). However, *I. scapularis* XIAP is substantially

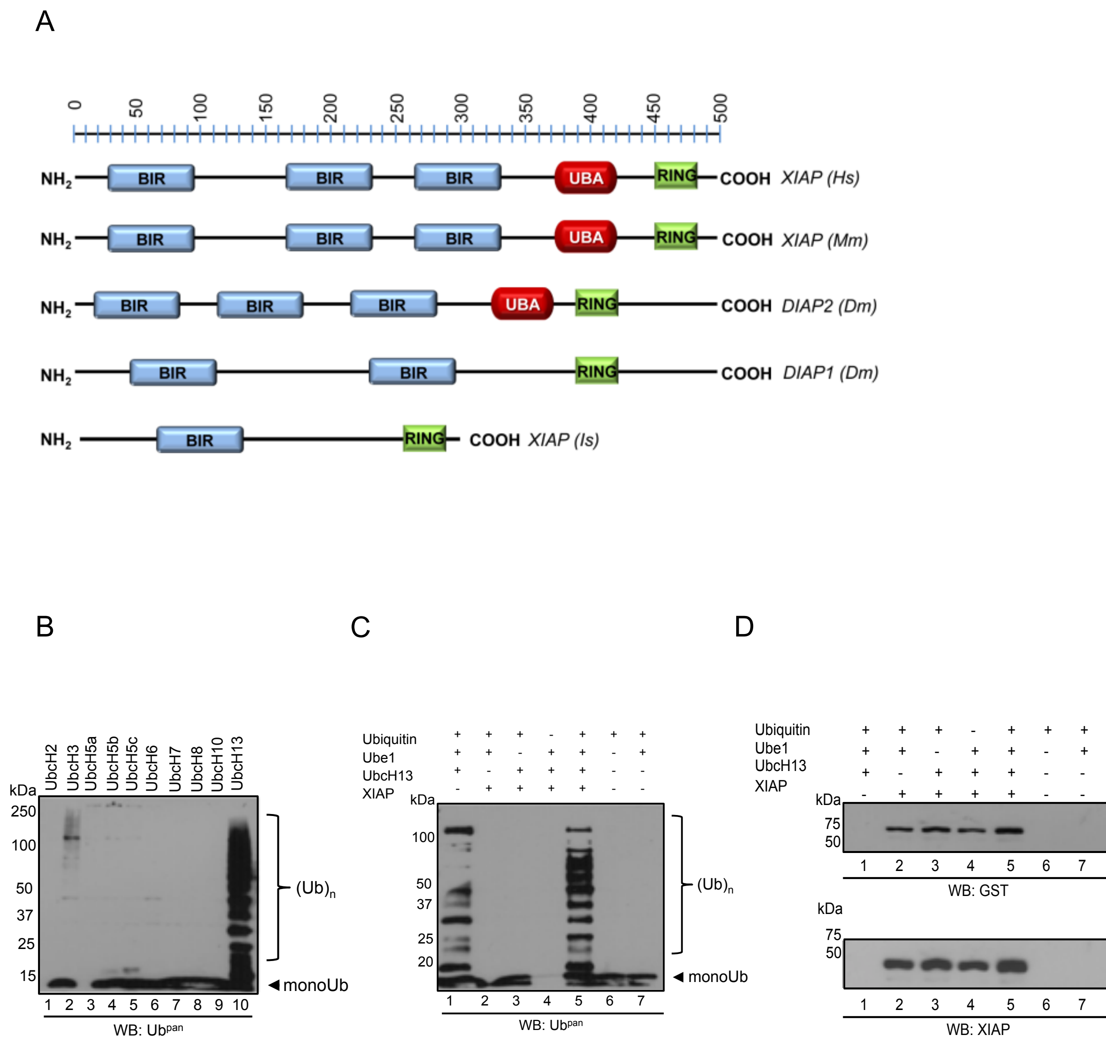


Figure 12: *I. scapularis* XIAP is an E3 ubiquitin ligase. (A) *I. scapularis* (Is) XIAP domains [(BIR - 61-135 aa. – blue); (RING – 255-289 aa. - green)] compared to related proteins in humans (Hs), mice (Mm) and *Drosophila* (Dm). UBA – ubiquitin associated domain (red). (B-C) Ubiquitination assays followed by Western blot. Polyubiquitination assays were performed in the presence of ubiquitin, an E1 ubiquitin-activating enzyme (Ube1), E2 ubiquitin-conjugating enzymes (UbcH) and the tick recombinant E3 ubiquitin ligase XIAP expressed in *E. coli*. Ten different E2 enzymes were used in (B) and UbcH13 was used as the E2 in (C-D). Aliquots were

resolved in 12% SDS-PAGE and probed with an Ub^{Pan} ubiquitin. Experiments were repeated at least twice. (D) Polyubiquitination assays were performed in the presence of XIAP expressed in *E. coli* tagged with GST. Aliquots were resolved in 12% SDS-PAGE and probed for GST (upper panel) and XIAP (lower panel).

shorter when compared to the mammalian and *Drosophila* proteins and does not carry two Baculoviral IAP Repeat (BIR) or the ubiquitin-associated (UBA) domains (Beug, Cheung et al. 2012). To address the role of *I. scapularis* XIAP in the context of ubiquitination, this protein was expressed and purified and assays with commercially available ubiquitin, E1 (Ube1) and E2 (UbcH) enzymes were performed. Recombinant *I. scapularis* XIAP derived from *Escherichia coli* was used because this system has been used for polyubiquitination assays (Fang, Jensen et al. 2000; Mace, Linke et al. 2008; Grant, Grant et al. 2012). Low levels of polyubiquitination were detected when XIAP was incubated with the E2 enzyme UbcH3 (Figure 12B, lane 2) and high levels of polyubiquitination when XIAP was combined with UbcH13 (Figure 12B, lane 10). Addition of ubiquitin in the absence of E1 (Ube1), E2 (UbcH13) and XIAP yielded only monoubiquitination (Figure 12C, lane 6). As previously observed, UbcH13 alone is capable of producing polyubiquitin due to autoubiquitination (Figure 12C, lane 1) (Doss-Pepe, Chen et al. 2005). However, addition of *I. scapularis* XIAP revealed increased quantity and diversity of polyubiquitin in the 50-100 KDa range, as judged by Ub^{pan} immunoblots (Fig. 2C, lane 5).

To demonstrate that the results obtained were not an UbcH13 artifact, more stringent experiments were performed. First, Ubpred (Radivojac, Vacic et al. 2010) was used to predict XIAP autoubiquitination sites. Although Ubpred predicted that XIAP may be autoubiquitinated (*data not shown*), no autoubiquitination activity was observed (Figure 12D). Immunoblots using two independent antibodies (GST tag or XIAP) showed that XIAP did not autoubiquitinate under the experimental conditions tested. Next, ubiquitins with lysine 63 (Ub^{K63R}) or lysine 48 mutated to arginine (Ub^{K48R}) were utilized to determine the type of linkages the *I. scapularis* XIAP is involved. Incubating XIAP with Ub^{K48R} did not show any alteration in activity (Figure 13A). On the other hand, polyubiquitination was not observed when XIAP was incubated with Ub^{K63R} ubiquitin (Figure 13B). As expected, incubation of XIAP and the wild-type ubiquitin (Ub^{WT}) showed polyubiquitination (Figure 13). Third, a dose-dependent polyubiquitination assay indicated that increased levels of *I. scapularis* XIAP enhanced polyubiquitination (Figure 13C, lanes 5-7). These results were confirmed with subsequent immunoblotting with Ub^{K63} and Ub^{K48} antibodies. Ub^{K48} polyubiquitination was not observed when Ub^{K48} immunoblots were performed (Figure 13C, lanes 8-10), whereas Ub^{K63} polyubiquitination increased with higher amounts of XIAP (Figure 13C, lanes 11-13). Importantly, ubiquitin chains were not observed when the Ub^{K63R} mutant was used, despite increased levels of XIAP (Figure 13C, lanes 14-16). Altogether, these findings provide strong evidence that *I. scapularis* XIAP carries out Ub^{K63}-linked polyubiquitination.

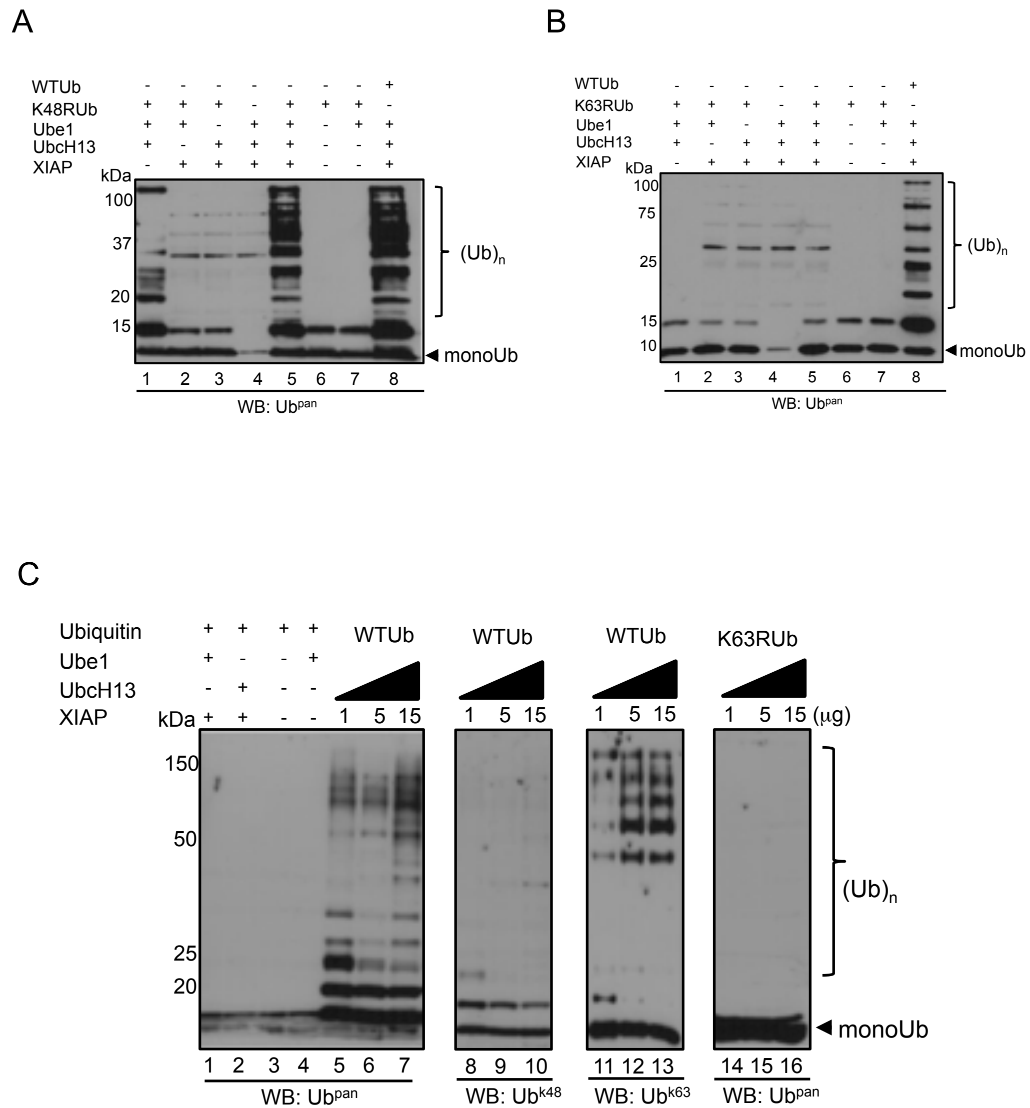


Figure 13: ***I. scapularis*** **XIAP promotes K63-linkage polyubiquitin chains.** Ubiquitination assays were performed and followed by Western blot. (A) K48RUb and (B) K63RUb were included in polyubiquitination assays. WT ubiquitin was used as a positive control. (C) WT and K63R ubiquitins were used. Aliquots were resolved in 12% SDS-PAGE and then probed with an Ub^{Pan} ubiquitin (lanes 1-7; 14-16), Ub^{K48} (lanes 8-10) and Ub^{K63}-specific antibodies (lanes 11-13). These experiments were repeated at least twice.

I. scapularis XIAP requires the RING domain for polyubiquitination

To gain additional insights into *I. scapularis* XIAP function, its catalytic RING domain was modeled based on the E3 ubiquitin ligase MDMX, a negative regulator of the tumor suppressor protein p53 (Wang and Jiang 2012). The *I. scapularis* XIAP RING domain consisted of 1 α-helix, 3 β-sheets and 5 loops that accommodated 2 structural zinc ions folding in a "cross-brace" fashion (Figure 14A). Of the residues in the RING domain, cysteine and histidine amino acids were the most conserved (*data not shown*). From the evolutionarily conserved amino acids (Ying, Huang et al. 2011), leucine, isoleucine and alanine) were retained but one hydrophobic amino acid was substituted by a serine (*data not shown*). Using antibodies specific for Ub^{K48}- and Ub^{K63}, XIAP polyubiquitination occured via Ub^{K63} but not Ub^{K48} residues (Figure 14B-C). To assess the role of the RING domain in polyubiquitination, *I. scapularis* XIAP without this domain (XIAP-ΔRING) was expressed and polyubiquitination assays performed. When the RING domain was deleted from *I. scapularis* XIAP, polyubiquitination activity was greatly diminished when compared to the wild-type XIAP (Figure 14C-D, lane 3). Importantly, a residual autoubiquitination activity of UbcH13 (Figure 14C-D, lane 1) was seen and the Ub^{K48R} mutant did not influence polyubiquitination by the wild-type XIAP (Figure 14C-D, lane 2). Conversely, wild-type *I. scapularis* XIAP catalysis was greatly influenced by the Ub^{K63R} mutant. *I. scapularis* XIAP catalysis did not occur when the Ub^{K63R} mutant was used (Figure 14C, lane 5).

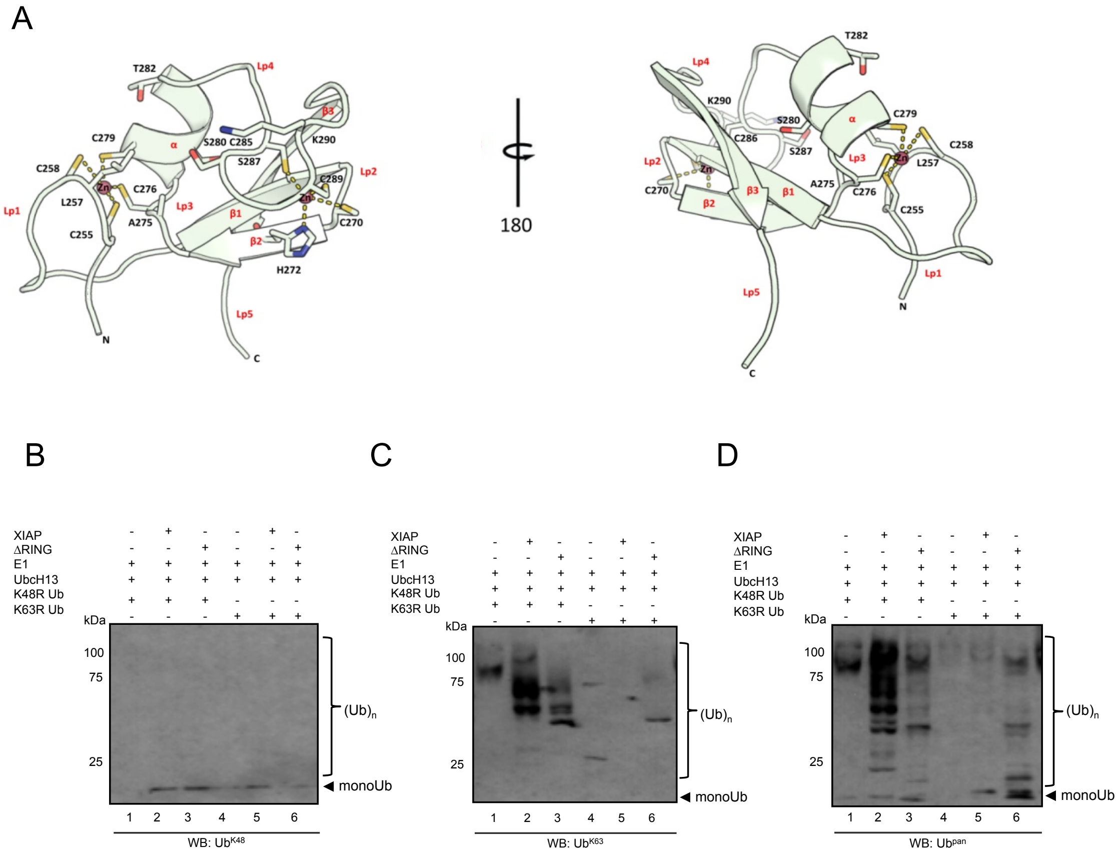


Figure 14: XIAP requires RING domain for polyubiquitination. (A) Two-views (180° rotated angle) of a ribbon diagram for the *I. scapularis* XIAP RING domain based on protein threading with the published MdmX (2vjf:D) structure. This model shows the characteristic cysteine-histidine "cross-brace" conserved motif where cysteines and histidines provide zinc (Zn) coordination sites for maintenance of the protein structure. Side chains of some amino acid residues are shown in stick format with oxygen labeled orange, nitrogen blue and sulfur yellow. α -helix, β-sheets and secondary loop structures are labeled in red, while conserved and consensus amino acids are written in black. Hydrogen bonds are shown in dashed yellow lines. (B-D) Polyubiquitination assays were performed using 3 μg XIAP and XIAP-ΔRING as ubiquitin ligases. Ubiquitin was replaced by Ub^{K48R} (lanes 1-3) or Ub^{K63R} (lanes 4-6) mutants. Reactions were immunoblotted

(WB) with (B) Ub^{K48}, (C) Ub^{K63} and (D) Ub^{pan} antibodies. These experiments were repeated at least twice.

RING domains have previously been shown to require two zinc cations to provide a stable structure for E3 ligases (Fang, Jensen et al. 2000). To determine whether *I. scapularis* XIAP was sensitive to zinc depletion, XIAP was incubated with the zinc chelator TPEN (Fang, Jensen et al. 2000) and subsequently rescued by the addition of $ZnCl_2$. When *I. scapularis* XIAP was subsequently probed with an antibody that recognizes Ub^{Pan} or Ub^{K63}, polyubiquitination activity was abrogated (Figure 15A-B, lane 4). Interestingly, XIAP-dependent polyubiquitination was readily restored in a $ZnCl_2$ concentration-dependent manner (Figure 15A-B, lanes 5-7). The sensitivity of *I. scapularis* XIAP to the alkylating agent NEM was also tested. NEM interacts with the sulfhydryl group of cysteine residues in certain E3 ligases, but not in RING-type E3 ligases, which are resistant to NEM activity (Lorick, Jensen et al. 1999; Fang, Jensen et al. 2000). As expected, incubation of *I. scapularis* XIAP with NEM prior to polyubiquitination assays had no effect on enzymatic activity (Figure 15C-D). Overall, these results suggest that *I. scapularis* XIAP requires zinc cations for polyubiquitination activity and the RING domain is essential for Ub^{K63}-type polyubiquitination.

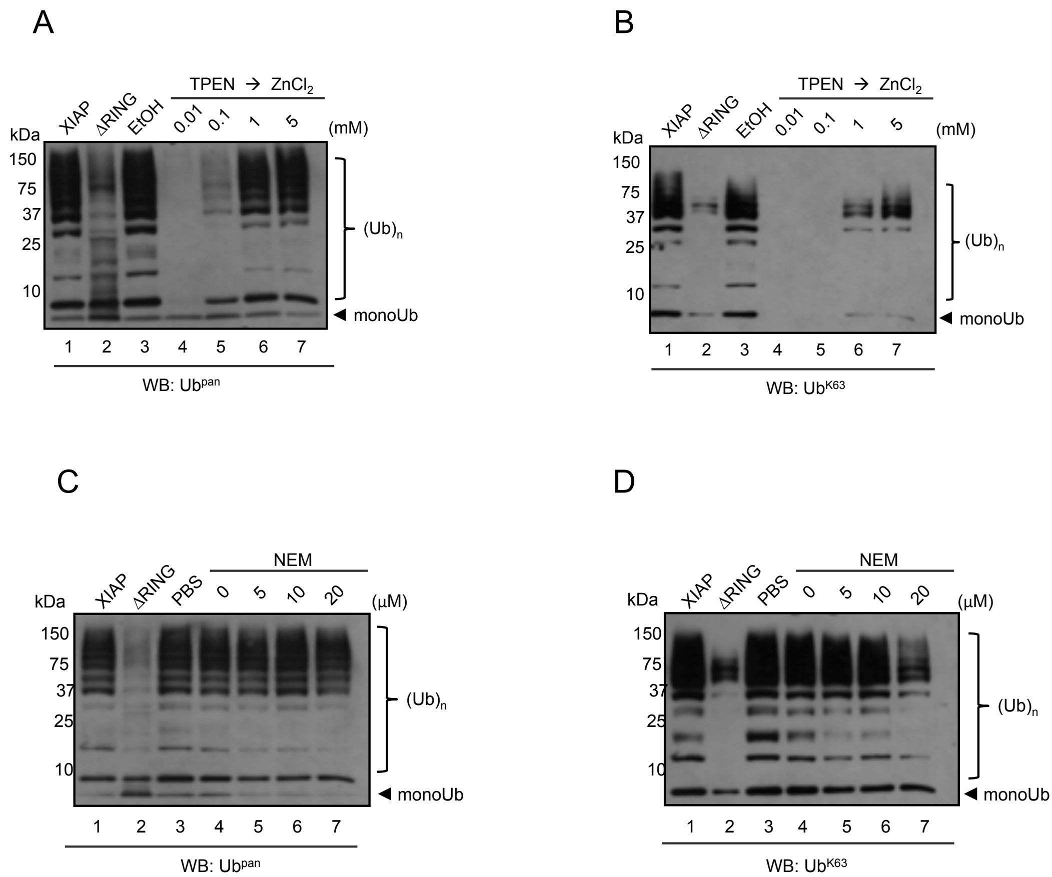
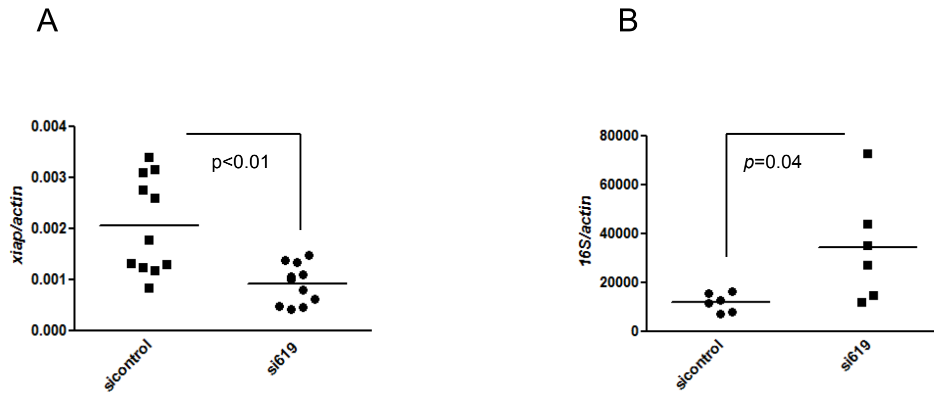


Figure 15: XIAP is resistant to NEM but sensitive to TPEN. (A-C) 2.5 μg of full length XIAP or XIAP-ΔRING were incubated with 2 mM TPEN at 4°C overnight. 0.5% ethanol was used as a mock control. Samples were then incubated with increasing amounts of $ZnCl_2$ (0.01 mM, 0.1 mM, 1 mM and 5 mM) for 45 minutes at room temperature. The total mixtures were used in polyubiquitination assays, resolved in 10% SDS-PAGE and immunoblotted with (A) Ub^{pan} and (B) Ub^{K63} antibodies. (C-D) 3 μg of XIAP or XIAP-ΔRING was incubated with increasing amounts of NEM (5 μM, 10 μM and 20 μM) for 30 min at room temperature. Samples were then used in polyubiquitination assays. Reactions were immunoblotted with (C) Ub^{pan} and (D) Ub^{K63} antibodies. These experiments were repeated at least twice.

XIAP restricts A. phagocytophilum colonization of I. scapularis

Because human XIAP was previously associated with *A. phagocytophilum* infection (Ge and Rikihisa 2006), siRNA was designed to determine whether the tick XIAP had any role in microbial pathogenesis. From two siRNAs designed, siRNA targeting the nucleotide positions 619-639 (si619) was proven to be the most successful (*data not shown*). *A. phagocytophilum* load in *xiap* silenced (si619) versus non-silenced ISE6 cells (sicontrol) was then compared (Figure 16A) *A. phagocytophilum* infection increased upon *xiap* silencing in ISE6 cells, as indicated by qRT-PCR and Romanowsky staining (Figure 16B-C). XIAP in mammals has been associated with apoptosis (Ge and Rikihisa 2006; Ribeiro, Kuranaga et al. 2007). Thus, *xiap* was silenced in ISE6 tick cells and cell death was verified. ATP quantification was used as a read-out for metabolically active cells because measuring cell death with standard mammalian lactate dehydrogenase (LDH) assays was hampered by high background levels - most likely due to the complexity of the ISE6 cell culture media. Importantly and similar to mammalian cells (Severo, Stephens et al. 2012), *A. phagocytophilum* inhibited cell death in tick cells at multiplicity of infection (MOI) 100 (Figure 16D). It was also observed that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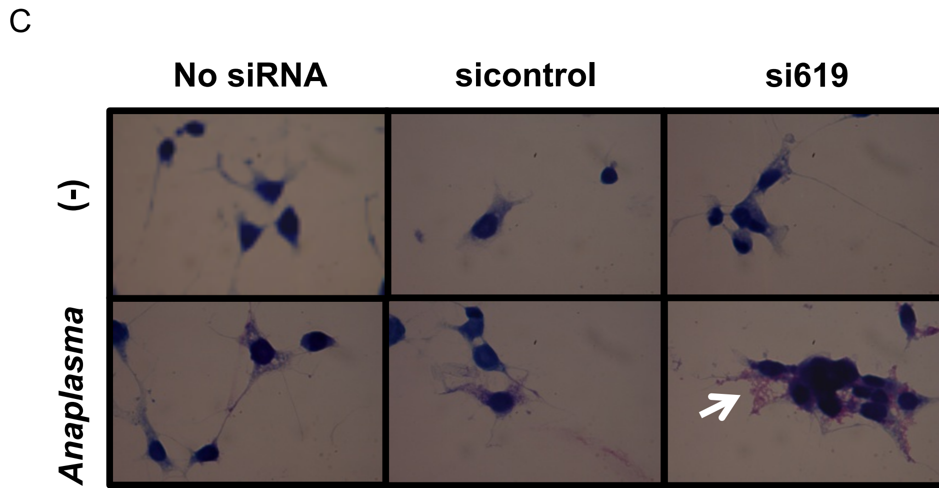
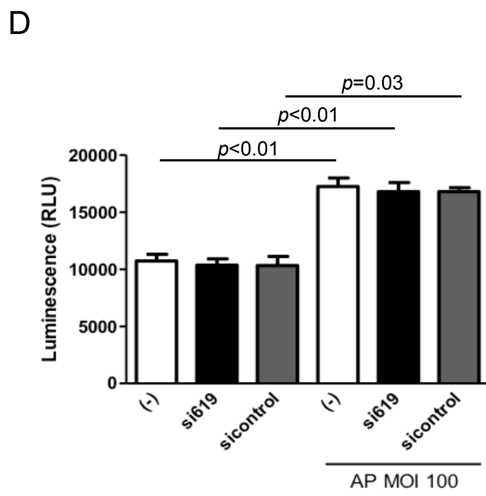
sicontrol
si619


C
No siRNA
sicontrol
si619
(-)
Anaplasma


D
p=0.03
p<0.01
p<0.01
20000
15000
10000
5000
0
Luminescence (RLU)
(-)
si619
sicontrol
(-)
si619
sicontrol
AP MOI 100

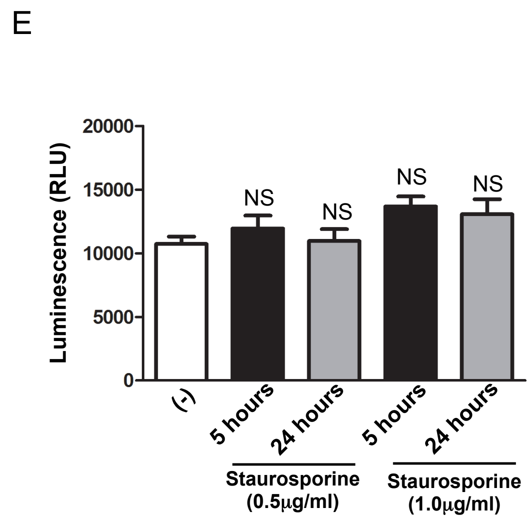

E
20000
15000
10000
5000
0
Luminescence (RLU)
NS
NS
NS
NS
(-)
5 hours
24 hours
5 hours
24 hours
Staurosporine (0.5μg/ml)
Staurosporine (1.0μg/ml)

Figure 16: XIAP silencing facilitates *A. phagocytophilum* colonization of ISE6 cells and does not affect cell death. (A) ISE6 *I. scapularis* cells (1 x 10^5) were transfected with *xiap* (si619) or scrambled siRNA (sicontrol) and *xiap* expression analyzed by qRT-PCR to confirm silencing. (B) *Xiap* (si619) or scrambled siRNA (sicontrol) transfected ISE6 cells (n=6) were infected with *A. phagocytophilum* (MOI 100) for 24 hours and *A. phagocytophilum* load was measured by qRT-PCR using the $\Delta\Delta^{Ct}$ method. (C) ISE6 cells (2X10^4) were stained by using kwik-diff, a commercial Romanowsky variant stain. *A. phagocytophilum* morulae are shown in purple (white arrow) while *I. scapularis* cells are shown in dark blue. (D) ISE6 *I. scapularis* cells (2X10^4) were transfected with siRNA 619 (600 ng) or siRNA control (600 ng) and infected with *A. phagocytophilum* (MOI 100) for 24 hours post-transfection. ATP presence signals cell viability and was measured as relative luminescence units (RLU). These experiments were repeated twice. (E) ISE6 *I. scapularis* cells (2X10^4) were treated with staurosporine at indicated concentrations and cell viability (as judged by ATP presence) was measured at described time points. Error bars represent standard error. Statistical analysis ($p<0.05$) was performed using Student's t test (A, B, D) and ANOVA (Bonferroni) (E).

xiap silencing did not affect cell death in *I. scapularis* ISE6 cells (Figure 16D), suggesting that this gene may not have an apoptotic role in *I. scapularis*. Alternatively, the redundancy of the *I. scapularis* genome (Pagel Van Zee, Geraci et al. 2007) could have "masked" the phenotype. This is reasonable because stimulation of *I. scapularis* ISE6 cells with different concentrations of staurosporine, a common trigger for mammalian cell apoptosis (Luhrmann and Roy 2007), did not induce cell death at 5 and 24 hours post-stimulation (Figure 16E).

I. scapularis nymphs were also microinjected with siRNAs. No difference in feeding (as judged by tick engorgement) was observed between ticks injected with *xiap* (si619) and control siRNAs (Figure 17A). This is important because it suggested that both groups of ticks were feeding similarly and *xiap* silencing did not influence engorgement. Silencing was obtained in tick salivary glands (Figure 17B) and midguts (Figure 17C). *A. phagocytophilum* load was also found to be higher in *I. scapularis* upon *xiap* silencing (Figure 17D-E). These findings suggest that *xiap* restricts *A. phagocytophilum* colonization of *I. scapularis* ticks.

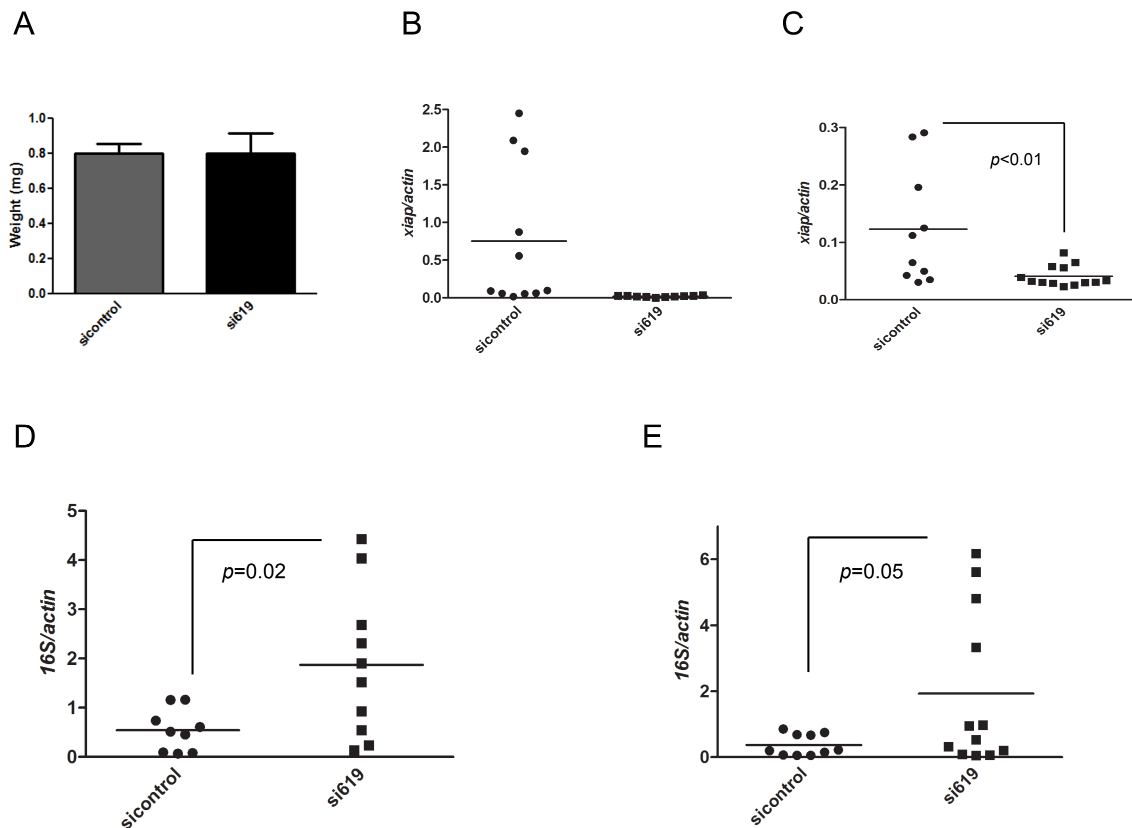


Figure 17: XIAP restricts *A. phagocytophilum* colonization of *I. scapularis*. (A-E) *I. scapularis* nymphs were body injected with 9.2 ηL containing 1 x 10^{13} molecules/μL of *xiap* siRNA (si619) or scrambled siRNA (sicontrol) and allowed to feed for 72h on *A. phagocytophilum* infected C57BL/6 mice. (A) Average weight of ticks (n=30) treated with sicontrol and si619 is shown. *Xiap* is silenced in (B) salivary glands (SG) and (C) midguts (MG). (D-E) *A. phagocytophilum* load was measured in the (D) SG and (E) MG by qRT-PCR using the $\Delta\Delta^{Ct}$ method for the 16S gene relative to tick β-actin expression. Each dot indicates individual or pools of two ticks (n=15 per group). Experiments were repeated twice. Error bars represent standard error. Statistical analysis was performed using the Student's t test (p< or equal to 0.05).

6.4 Concluding remarks

How polyubiquitination regulates pathogen colonization of medically-relevant arthropods has not yet been determined. Here, a tick E3 ubiquitin ligase, named XIAP, was described as restricting bacterial colonization of an arthropod vector. Polyubiquitination has been widely demonstrated to regulate microbial pathogenesis and immunity (Jiang and Chen 2012; Vandenabeele and Bertrand 2012). For example, nuclear factor (NF)-κB activation is controlled by polyubiquitination in MyD88-dependent pathways following exposure to pathogens (Vandenabeele and Bertrand 2012). The E3 ubiquitin ligase TRAF6 is also recruited when Toll-like receptors (TLRs) are activated, leading to Ub^{K63} polyubiquitination of kinases (Jiang and Chen 2012). This mechanism appears evolutionarily conserved because Ub^{K63} polyubiquitination of the DREDD caspase and the immunodeficiency (IMD) molecule requires DIAP2 during infection of

Drosophila (Silverman, Paquette et al. 2009). *I. scapularis* XIAP and DIAP2 share similarities and our results indicate that the tick XIAP catalyzes Ub^{K63} polyubiquitination via the RING domain, despite the absence of an UBA domain. This corroborates with findings that illustrated that the mammalian XIAP does not require the UBA domain for E3 ligase activity (Gyrd-Hansen, Darding et al. 2008).

It is possible that XIAP-mediated Ub^{K63} polyubiquitination may regulate immune signaling during *A. phagocytophilum* colonization of ticks. This hypothesis is supported by increased *A. phagocytophilum* acquisition of *I. scapularis* after *xiap* silencing. Interaction between XIAP and UbcH13, a protein that shares strong similarities with bendless in *I. scapularis* (E value < 2^{e-96}) and a modulator of the IMD pathway in arthropods (Meinander, Runchel et al. 2012), reiterates our reasoning. It is unclear how RING domains of E3 ubiquitin ligases transfer ubiquitin to substrate proteins. It is suggested that a dimeric XIAP RING domain is necessary for polyubiquitination activity (Feltham, Khan et al. 2012). Though not yet proved, there is some evidence of XIAP dimerization during *A. phagocytophilum* infection of ISE6 cells. XIAP dimerization appears very strong because attempts to rupture this dimer under different conditions were unsuccessful.

I. scapularis XIAP was not found to be autoubiquitinated. This is contrary to reports observed for XIAP homologues in mammals, where autoubiquitination and proteasomal degradation seems to be a requirement for apoptosis (Beug, Cheung et al. 2012). Although no effect of *I. scapularis* XIAP on cell death was observed, the possibility that *I. scapularis* XIAP may perform autocatalytic functions under physiological conditions, as many E3 ligases require accessory proteins for their activity,

should not be excluded (Hoeller and Dikic 2009; Collins and Brown 2010; Harhaj and Dixit 2011; Beug, Cheung et al. 2012). As previously shown, *I. scapularis* XIAP share similarity with MdmX and this protein interacts with another E3 ubiquitin ligase named Mdm2 through its RING domain (Wang and Jiang 2012). Undoubtedly, clarifying the physiological role of XIAP during pathogen infection of ticks will be important. However, this endeavor is not currently possible because the technology to insert or delete genes in ticks is not available.

Understanding the polyubiquitination machinery may allow for the development of innovative strategies to treat vector-borne illnesses. It would be fascinating to apply chemical screening assays with the intent of modulating the arthropod ubiquitome. This approach would be a first step towards the development of structure-based molecules that target vector-pathogen interactions. This is not unreasonable as pharmacological inhibitors named second mitochondria-derived activator of caspase (SMAC) mimetics have been successfully used in *Drosophila* (Chew, Chen et al. 2009). Hence, SMAC mimetics may provide novel therapeutic opportunities for the treatment of vector-borne diseases. In summary, the results presented here promote a significant advancement in ubiquitin biology in the context of pathogen colonization of medically relevant arthropod vectors.

6.5 Materials and methods

Ethics statement

The Institutional Animal Care and Use Committee approved all experiments. C57BL/6 mice (6-10 weeks) were purchased from Jackson Laboratories. *I. scapularis* nymphs were obtained from Oklahoma State University and reared at 23°C with 85% relative humidity and 14 hour light/10 hour dark cycle. Experimentation with *A. phagocytophilum* (HZ strain) was approved by the Biological Use Authorization Committee (BUA number: 20120020). *A. phagocytophilum* was grown in HL-60 cells, as described (Chen, Severo et al. 2012).

Bioinformatics analysis

Prediction of XIAP domains was done according to ExPASy (http://us.expasy.org). Structural modeling of the *I. scapularis* XIAP RING domain was done based on the Swiss-PROT homology modeler (Bordoli, Kiefer et al. 2009). Images were generated with PyMOL (Schrodinger Licensing). Ubiquitination sites were predicted using Ubpred (Radivojac, Vacic et al. 2010).

ISE6 cells and viability assays

The *I. scapularis* cell line ISE6 was a gift from Dr. Ulrike Munderloh at the University of Minnesota and was maintained, as described (Munderloh, Liu et al. 1994).

3 µg of siRNA were added to 9 µl RNAiFect (Qiagen) in 100 µl of L15C-300 (Leibowitz) cell culture medium, incubated at room temperature for 15 minutes and then diluted in 400 µl of fresh medium. Control cells received equivalent concentration of scrambled siRNA and plates were maintained at 34°C for at least 24h. RNA was extracted in TRIzol (Invitrogen), and cDNA was prepared using Verso (Thermo Scientific). Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed using the iQ SYBR green supermix (Bio-Rad) and gene-specific primers (Table 2). Cell death assays were performed using the Cell Titer-Glo® Luminescent Cell Viability Assay (Promega), which quantifies ATP production. Staurosporine (Sigma-Aldrich) was used at indicated concentrations.

SiRNA synthesis

Small interfering RNA (siRNA) target sites were chosen based on the Invitrogen website (www.invitrogen.com). The sequence for *xiap* was compared to the *I. scapularis* genome (www.vectorbase.org) (Lawson, Arensburger et al. 2007; Lawson, Arensburger et al. 2009) and the National Center for Biotechnology Information (NCBI) database (Benson, Karsch-Mizrachi et al. 2011) using Basic Local Alignment Search Tool (BLAST) to eliminate homologies. *Xiap* siRNA sequences (Table 2) were synthesized using the silencer siRNA construction kit (Ambion).

I. scapularis siRNA microinjection

10-15 *I. scapularis* nymphs were held with forceps and microinjected (Nanoject II, Drummond Scientific) in the abdomen at 45 degrees and a 46nl/sec injection rate, with 9.2 ηL containing 1 x 10^{13} molecules/μL of *xiap* or scrambled siRNAs. *I. scapularis* were left to rest for 30 minutes to 2 hours and allowed to feed for 72 hours on *A. phagocytophilum*-infected C57BL/6 mice. Nymphs were then dissected under the microscope and salivary glands or midguts were processed either individually or in pools of two for analysis.

Confocal microscopy

Tick-immune and anti-*A. phagocytophilum* rabbit polyclonal sera were described (Narasimhan, Sukumaran et al. 2007). Confocal microscopy was done using a Leica SP2 microscope. ISE6 cells were seeded onto cover slips treated with 2% gelatin. Ticks were dissected in phosphate buffered saline (PBS) and allowed to dry on slides (Colorfrost Plus, Fisher). Cover slips were fixed in 2% paraformaldehyde for 20 minutes at room temperature. Slides were fixed in 4% paraformaldehyde for 1 hour at room temperature. Slides and/or cover slips were stained with anti-*A. phagocytophilum* (1:100), anti-Ub^{K48}, anti- Ub^{K63} and Ub^{pan} antibodies (1:50) (Boston Biochem) and an anti-rabbit Fluorescein isothiocyanate (FITC) secondary antibody (1:200). 4',6-diamidino-2-phenylindole (DAPI) was used to stain the nucleus while Evans blue stained the cells. ISE6 cells were also stained using Romanowsky staining (Kwik diff system, Thermo Scientific).

XIAP cloning and expression

TOPO cloning strategy was chosen to clone *I. scapularis* XIAP and XIAP-ΔRING. The PGEX-6P-2 plasmid (GE Healthcare) was used to transform the *E. coli* BL21 Gold (DE3) strain (Agilent) and express XIAP and XIAP-ΔRING. Expression was induced with isopropylthio-β-galactoside (IPTG) and purification and solubilization were performed, as described (Frangioni and Neel 1993).

OligoPerfect^{TM} (Invitrogen) was used to design primers (Table 2) for our TOPO cloning strategy to clone *I. scapularis* XIAP and XIAP-ΔRING. Amplicons were ligated into pCR®2.1-TOPO® and *E. coli* TOP10 strain (Invitrogen) was transformed. *EcoRI* and *NotI* restriction sites were added to *I. scapularis* XIAP and ΔRING-XIAP amplicons for subcloning. Amplicons were digested with *EcoRI* and *NotI* high-fidelity restriction enzymes (New England BioLabs) and ligated into the digested *EcoRI* and *NotI* PGEX-6P-2 plasmid (GE Healthcare). XIAP and ΔRING-XIAP PGEX-6P-2 constructs were then used to transform the *E. coli* BL21 Gold (DE3) strain (Agilent).

Expression was induced with IPTG (0.1 mM) at 20°C for 20 hours. Briefly, *E. coli* BL21 Gold (DE3) strain induced with IPTG was pelleted by centrifugation (3,220 x g, 15 minutes, 4°C) and washed with sodium chloride and Tris-ethylenediaminetetraacetic acid (EDTA) (STE) buffer (150 mM NaCl, 10 mM Tris, pH 8.0, 1 mM EDTA). Pellets were re-suspended in 1 mg/ml lysozyme in STE buffer and incubated for 1 hour at 4°C with rotation. Dithiothreitol (DTT) was added to a final concentration of 5 mM. Bacteria were lysed by the addition of 1.5% N-laurylsarcosine (sarkosyl). Lysates were obtained by centrifugation (3220 x g, 4C, 20 min). Supernatant was taken and Triton X-100 added

to a concentration of 2%. Glutathione-S-transferase (GST) beads (BD Biosciences) were added and incubated on a rotator at 4°C overnight. Beads were washed with cleavage buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM, EDTA, 1 mM DTT) and the GST fusion XIAP was cleaved with PreScission protease (GE Healthcare) for 4 hours at 4°C.

Ubiquitination assays

Ubiquitination assays were performed by combining 3 μg of *I. scapularis* GST-XIAP, XIAP or XIAP-ΔRING with 0.3 μg E1, 0.1 μg of E2 enzymes, 5 μg of ubiquitin, 1.5 μl of 10x Energy Regeneration Solution (ERS) and 2.5 μl of polyubiquitination buffer (50 mM Tris-HCl, p.H. 7.4, 1 mM DTT, 200 μM $ZnCl_2$) (Boston Biochem). Reactions were carried out for 2 hours at 35°C. Samples were heated at 95°C in SDS-PAGE 4x sample buffer (Bio-Rad) before loading onto 10% SDS-PAGE. Proteins were blotted onto 0.2 μm nitrocellulose membrane (Bio-Rad). Immunoblots were probed with primary antibodies at 4°C overnight (1:2500 Ub^{pan} dilution, 1:1000 Ub^{K48} dilution, 1:250 Ub^{K63} dilution) (Millipore, Billerica, MA). Antibody specificity was assessed by pre-incubating the antibodies with 4 μg of either $Tetra^{K48}$ or $Tetra^{K63}$-linked ubiquitin for 1 hour. Custom-made *I. scapularis* XIAP antibodies were obtained (Thermo Scientific). XIAP (1:500 dilution) and GST (1:500 dilution) (Calbiochem, Millipore) antibodies were used for autoubiquitination studies. Secondary antibodies were used at a 1:8000 dilution. Blot was covered with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Immunoblots were stripped using Multi-Western Stripping Buffer (Bioland Scientific). For Zn chelation, 2.5 μg of XIAP were incubated with 2 mM tetrakis-(2-

pyridylmethyl) ethylenediamine (TPEN) (Sigma-Aldrich) or 0.5% ethanol (mock) overnight at 4°C. Samples were then treated with indicated amounts of $ZnCl_2$ for 45 minutes at room temperature. For alkylation experiments, 3 μg of XIAP were treated with indicated concentrations of N-ethylmaleimide (NEM) (Sigma-Aldrich) in PBS for 30 minutes at room temperature.

Statistical analysis

Data were expressed as means ± standard errors of the means (SEM). D'Agostino-Pearson omnibus test ,unpaired Student's *t* test and one-way analysis of variance (ANOVA), followed by Bonferroni post hoc multiple-comparison tests were used. Analyses were performed using GraphPad Prism 5.04. P < or equal to 0.05 was considered statistically significant.

Table 2. Primer sequences

Gene	Accession Number	Sequence
Anaplasma 16S (qRT-PCR)	NC_007797	F 5’-CAGCCACACTGGAACTGAGA-3' R 5'-CCCTAAGGCCTTCCTCACTC-3'
Ixodes Actin (qRT-PCR)	AF426178	F 5’-GGTATCGTGCTCGACTC-3’ R 5’-ATCAGGTAGTCGGTCAGG-3’
Ixodes XIAP (qRT-PCR)	XM_002433822	F 5’-CAGAGCAATGGACAGCCTTT-3’ R 5’-CTCTGGATCCCCCTTGAACT-3’
XIAP-TOPO		F 5’-GTTGTCATCAGCATGGCG-3’ R 5’-TCATGAAAGAAAAGCCTTAAT-3’
XIAP-TOPO-ΔRING		F 5’-GTTGTCATCAGCATGGCG-3’ R 5’-GCAGCGAGAGTCCGTGG-3’
XIAP-PGEX-6P-2		F 5’-GgaattcCCGTTGTCATCAGCATGGCG-3’ R 5’-AAGGAAAAAAgcggccgcTCATGAAAGAAAAGCCTTAAT-3’
XIAP-PGEX-6P-2-ΔRING		F 5’-GgaattcCCGTTGTCATCAGCATGGCG-3’ R 5’-AAGGAAAAAAgcggccgcGCAGCGAGAGTCCGTGG-3’
619siRNA		F 5'-AAGCTACTCATCTCTCGAGGTCCTGTCTC-3' R 5'-AAACCTCGAGAGATGAGTAGCCCTGTCTC-3'
619sicontrol		F 5’-AAGTCGCTACAGTCCGTTACTCCTGTCTC-3’ R 5’-AAAGTAACGGACTGTAGCGACCCTGTCTC-3’
781siRNA		F 5’-AACGATCGCCAGGTGATCTTTCCTGTCTC-3’ R 5’-AAAAAGATCACCTGGCGATCGCCTGTCTC-3’
781control		F 5’-AAGTCGCGGCTTCCGATATTACCTGTCTC-3’ R 5’-AATAATATCGGAAGCCGCGACCCTGTCTC-3’

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Chapter 7: Conclusion

Here, the multiple angles of vector-borne diseases were discussed using the tick-borne pathogen *A. phagocytophilum* as a model. Ticks, as well as other important arthropod vectors, were described in the context of their immune response against medically significant human pathogens. This is highly significant considering that the vector immune defense against a human pathogen dictates the maintenance and further transmission of such pathogen within the vector-borne disease cycle. During the last several years, there have been significant advances in our understanding of the cellular and biochemical events controlling the establishment of microbial populations within the arthropod vector. Immune evasion and manipulation of intracellular signaling pathways by arthropod-borne pathogens have been demonstrated and many more mechanisms underlying these interactions are expected to be soon unraveled.

In this thesis, the tick E3 ubiquitin ligase XIAP was demonstrated as critical for *A. phagocytophilum* colonization of its main arthropod vector, *I. scapularis*. This is the first report of an E3 ubiquitin ligase that controls colonization of medically relevant vector of a human pathogen. XIAP was also shown to promote K63 type of polyubiquitination *in vitro* and require zinc for catalysis. One may speculate that, perhaps, K63 polyubiquitination of intracellular molecules could lead to the activation of cascades related to apoptosis inhibition *in vivo*. Another possibility is that K63 polyubiquitination of target proteins would result in a downstream up-regulation of antimicrobial peptides. In the absence or at lower levels of such molecules, *A. phagocytophilum* would be rendered free to replicate and, hence, be found at higher numbers in midguts and salivary glands of *I. scapularis*. Further studies are required to determine how polyubiquitination affects *A. phagocytophilum* infection of the tick host. Analysis of *A.*

phagocytophilum transmission upon *xiap* knockdown could also provide researchers with a broader view of the tick-pathogen-host cycle.

In the interface between pathogen transmission and mammalian host colonization by vector-borne pathogens lies the vector's saliva. In many cases, pathogen transmission occurs during blood feeding, and arthropod saliva is one of the many components influencing such occurrence. Tick saliva has immunomodulatory, anesthetic and anti-coagulation roles. An effect of saliva in modulating cytokine secretion by mammalian cells upon infection with a vector-borne pathogen was illustrated here. More precisely, one tick salivary protein – sialostatin L2 – was demonstrated as an immunomodulator of host immune response through caspase-1 signaling inhibition. This is a major contribution to the scientific community since it corresponded to the first report of a tick salivary protein affecting the inflammasome pathway. The mammalian immune-derived pathology upon infection with *A. phagocytophilum* was also described to depend on a dihydrolipoamide dehydrogenase 1 (LPDA1) enzyme. Infection with an *A. phagocytophilum lpda1* mutant caused an altered hematological profile compared to infection with the *A. phagocytophilum* wild-type. One may hypothesize that LPDA1 is an effector molecule contributing to the ability of *A. phagocytophilum* to further colonize neutrophils, their major host cells.

Taken together, the results illustrated here contribute to our understanding of the numerous events controlling the vector-borne disease cycle. More mechanistic analyses describing these and other processes in greater depth, in addition to more holistic approaches to the vector-host-pathogen triad are needed to promote the advancement of the field of vector-borne diseases.